Universidad de Alcalá

Facultad de Química

Departamento de Química Analítica e Ingeniería Química

Nuevas estrategias miniaturizadas basadas en Inmunoanálisis Electroquímico soportado sobre partículas magnéticas para la detección y el control de la micotoxina Zearalenona

Tesis Doctoral

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CERTIFICAN:

Que el trabajo descrito en la presente memoria, titulado **"NUEVAS ESTRATEGIAS MINIATURIZADAS BASADAS EN INMUNOANÁLISIS ELECTROQUÍMICO SOPORTADO SOBRE PARTÍCULAS MAGNÉTICAS PARA LA DETECCIÓN Y EL CONTROL DE LA MICOTOXINA ZEARALENONA"**, ha sido realizado bajo su dirección por Dña. Mirian Hervás Yela en el laboratorio de Química Analítica de la Facultad de Química de esta Universidad. Asimismo, autorizan su presentación para que sea defendido como Tesis Doctoral.

Y para que conste y surta los efectos oportunos, firman el presente en Alcalá de Henares a 25 de Mayo de 2011.

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"Es preferible nadar en quimeras a esperar en la orilla"

(Quique González)

Summary

In the project of this Thesis, the mycotoxin ZEA has been chosen as the target analyte for the development of various analytical strategies, based on Electrochemical Immunoassay, to improve its identification and control in food samples. These analytical strategies intend to configure an alternative and competitive route to the existing ones, which employ expensive and sophisticated analytical instrumentation.

Immunoassays, mainly ELISA methods, have proven to be an excellent technology as sensitive, specific, without the need of extensive clean-up, rapid, simple and with a high throughput for the detection of different chemicals in a wide variety of food matrices including mycotoxin analysis.

Moreover, electrochemical detection (ED) is a valuable tool because of its high sensitivity, simplicity, suitability for mass-production and low cost. Besides, in immunoassays, ED is one of the most appropriated tools due to the large number of labels (enzymes and electroactive molecules) and the good behaviour of enzymatic reactions coupled to charge transfer reactions. Additionally, in the field of miniaturization and microfluidic systems, ED appears as a very suitable alternative to optical detection due to its inherent miniaturization and compatibility with microfabrication techniques without loss of performance.

By other hand, zearalenone (ZEA), a nonsteroidal oestrogenic mycotoxin produced by several species of *Fusarium*, can be found in different cereals and it has attracted recent attention because of the adverse effects on human and farm animal's health. A maximum tolerable amount of 20 μg Kg^{-1} has been set in baby food. This fact has made mycotoxin analysis, and particularly ZEA monitorization, one of the most important fields of food analysis. The extremely low level of this mycotoxin requires from the development of new sensitive and specific methods for rapid detection of ZEA in food samples, below the current and forthcoming more restricted maximum tolerable amounts.

The design of this Thesis was configured from the initial development of a conventional ELISA methodology with electrochemical detection, towards new strategies situated in the

more contemporary scene of the Analytical Chemistry comprising miniaturized, simplified and automatized systems. These new strategies include the development of an electrochemical immunosensor on screen-printed disposable platforms and a full-integrated electrochemical immunoassay into a lab-on-a-chip device.

The fundamental approach relays on common Enzyme-linked immunosorbent assay (ELISA) performed in conventional microwells, and coupled to electrochemical detection accomplished on the surface of carbon screen-printed electrodes. Basically, the immunoassay procedure was developed on the basis of a competitive scheme, where the mycotoxin ZEA and an enzyme-labelled derivative compete for the binding sites of the specific antibody. Protein G covalently bound to magnetic particles acts as an oriented immobilization support for the capture of the anti-ZEA antibody. After the molecular recognition event takes places, the extent of the affinity reaction is evaluated by the sensitive detection of a redox mediator directly related to the activity of the enzyme tracer (Horseradish peroxidase-ZEA).

All parameters and conditions involved in this basic methodology were previously optimized, since they constitute the basis for the next and more relevant strategies. Evaluation of the analytical performance was mainly focused on the detection limit and accuracy. A remarkable detection limit, which is comparable with the best one described in the literature, and excellent accuracy, evaluated using a certified reference material (CRM), shows the potential of the proposed system as a valuable tool for the sensitive detection of ZEA in baby food. The applicability for real sample analysis was confirmed by the analysis of representative infant food samples containing cereals as a liquid milkshake and a powdered baby food.

From this successful starting methodology and taking in account the extraordinary properties offered by biosensors technology, the next approach was focused in the development of an electrochemical immunosensor based on the use of magnetic beads and disposable carbon screen printed electrodes (CSPE). Transfer of the previous developed methodology onto the surface of screen-printed electrodes, allows the possibility of producing a disposable and point-of-care analytical system. In this case, and after biological

recognition, the immunocomplex-modified paramagnetic beads were confined on the surface of CSPE by the aid of a magnet, where electrochemical detection can be easily completed. Benefits of this portable methodology can be obtained, while keeping an excellent LOD and accuracy similar to the initial strategy. Additionally, and in order to diminish the time consuming and laborious procedure for routinely performing a four parameters logistic calibration curve, a simplified calibration protocol is also proposed. Sequential calibration and analysis of target mycotoxin, using just one disposable CSPE each, can be accomplished. Excellent analytical features in terms of accuracy and precision were again obtained with a remarkable low systematic error (less than 4%) and excellent reproducibility (RSD=6%). This strategy enhances the analytical merits of the electrochemical immunosensor as a very promising disposable analytical tool for *in situ* food-safety diagnosis.

The second option explored in this Thesis, lies with the combination of electrochemical immunoassays and microfluidic platforms. This strategy makes use of the benefits provided by microfluidic chips such as faster analysis times, extremely low sample and reagent volumes, parallelization of analysis into a small monolithic piece, together with being an ideal platform for performing microscale flow injection analysis with accurate fluid control and manipulation capabilities.

In a previous step towards the total integration of electrochemical immunoassay into a microfluidic platform, a relevant work has been performed for electrochemical monitorization *on-chip* of the enzymatically-generated product derived from the ELISA methodology. After immunoassay was performed *off-chip* in single ELISA microwells, the enzymatic product was electrokinetically injected and pumped for electrochemical detection *on-chip*. Simplified, fast and sequential analysis and calibration can be accomplished using a multiplexed format within the microfluidic chip. An extremely low sample concentration of less than 1 ppb was easily determined, allowing the control within regulatory limits of ZEA in baby foods.

The final option presented, which can be seen as the ultimate aim of this Thesis, deals with the full integration of the different steps regarding to biological recognition and

electrochemical detection on a single microfluidic device. The multiple benefits derived from using magnetic microbeads (large available surface area, easy manipulation for different steps and reloading), micrometer-sized channels (less sample and reagents volumes, reduced diffusion length for biological interactions), and electrochemical detection, greatly improve the efficiency of the immunoassay.

This strategy implies the creative use of the simple channel layout of the double-T microchip to perform sequentially the immunointeraction and enzymatic reaction within the microchip. Applying a program of electric fields suitable connected to the reservoirs for driving the fluidics at different chambers, allows performing the different reactions. The system is able to determine the ZEA mycotoxin lowering the total immunoassay dedicated time (15 minutes), avoiding manual steps in the procedure and using only a few tens of nanoliters of reagents. Additionally, a suitable detection limit of 0.4 ppb, low systematic error of 2% from the analysis of the CRM and excellent recoveries of 103 and 101 % for solid and liquid samples respectively, were obtained.

Resumen

En este trabajo de Tesis se ha llevado a cabo el desarrollo de diversas estrategias analíticas, basadas en inmunoanálisis electroquímico, aplicadas a la identificación y control de una micotoxina (zearalenona) en muestras alimentarias. Estas metodologías se erigen como una importante y competitiva alternativa a las técnicas clásicas que suelen hacer uso de instrumentación cara y sofisticada.

En los últimos años, los métodos inmunoanalíticos se han establecido como la metodología de elección para la determinación de micotoxinas en general, y de ZEA en particular, gracias a las excelentes características que ofrecen. Entre ellas, destacan su elevada especificidad, sensibilidad, relativa rapidez, sencillez y bajo coste.

Por otra parte, la detección electroquímica (ED) se configura como una valiosa herramienta debido a su alta sensibilidad, fácil utilización y costes relativamente bajos. Además, en el caso de los métodos inmunoanalíticos, es una herramienta idónea debido a la gran cantidad de marcadores (enzimas y moléculas electroactivas) y al buen comportamiento de las reacciones enzimáticas acopladas con reacciones de transferencia de carga. Es sin embargo, en el campo de la miniaturización y de los sistemas microfluidicos, donde la detección electroquímica demuestra claramente un gran potencial y aparece como la más importante alternativa a la detección óptica, debido a su miniaturización inherente sin pérdida importante de sensibilidad y, sobre todo debido a su compatibilidad con las técnicas de microfabricación.

El analito elegido, zearalenona (ZEA), es una micotoxina no esteroidea con actividad estrogénica producida por varias especies del género *Fusarium.* Esta micotoxina aparece en la mayoría de los cereales de grano pequeño y, en los últimos años, ha sido objeto de gran atención debido a los efectos adversos que supone tanto para la salud humana como animal. La cantidad máxima permitida de ZEA, tanto en alimentos destinados al consumo humano como para animales, varía de unos países a otros siendo 20 μg Kg^{-1} (ppb) el límite más restrictivo aplicado a alimentos infantiles. Debido a la toxicidad que conlleva su presencia en matrices alimentarias, su detección se ha convertido en uno de los campos más importantes dentro del análisis de alimentos. Además, teniendo en cuenta los bajos niveles

de concentración a los que se encuentran las micotoxinas y con objeto de mejorar su regulación y control en alimentos, ha surgido la necesidad de desarrollar métodos cada vez más sensibles y específicos que permitan su adecuada determinación por debajo de los límites exigidos por la legislación.

El diseño de esta tesis tiene como punto de partida el desarrollo de un método ELISA convencional con detección electroquímica, a partir del cual se evoluciona de forma conceptual y natural hacia dos estrategias miniaturizadas situadas en la escena más contemporánea de la Química Analítica, la cual tiende a la simplificación, miniaturización y automatización de estos sistemas. Estas nuevas estrategias incluyen el desarrollo de un inmunosensor electroquímico sobre electrodos serigrafiados de carbono desechables y la integración total del ELISA electroquímico en una plataforma microfluídica.

La aproximación fundamental se basa en el desarrollo de un método ELISA convencional en placa, acoplado a su detección electroquímica en la superficie de electrodos serigrafiados de carbono. El inmunoensayo se desarrolla en base a un esquema competitivo directo, en el que la micotoxina ZEA y el conjugado enzimático (ZEA-HRP) compiten por los sitios de unión del anticuerpo específico para ZEA. En este trabajo se han utilizado partículas magnéticas recubiertas de proteína G como soporte sólido que permite la inmovilización orientada del los anticuerpos anti-ZEA. Una vez que ha tenido lugar el reconocimiento molecular (reacción antígeno-anticuerpo), la determinación de ZEA se basa en la detección amperométrica de mediador electroquímico (benzoquinona), directamente relacionada con la actividad de la enzima (HRP) que ha quedado retenida por la interacción del conjugado enzimático con el anticuerpo.

Todos los parámetros y condiciones involucrados en esta metodología de partida fueron previamente optimizados, y constituyen la base de las siguientes estrategias. Asimismo, se ha llevado a cabo la evaluación del método bajo el marco de la legislación vigente tomando como referencia las dos propiedades analíticas que hacen válida a dicha estrategia para el control de seguridad en alimentos: el límite de detección (LOD) y la exactitud. Los resultados obtenidos pueden considerarse excelentes, tanto respecto al límite de detección, comparable con el mejor descrito en la literatura, así como la exactitud, evaluada frente a un

material de referencia certificado (MRC); en ambos casos, demostrando el potencial del sistema propuesto como una herramienta valiosa para la detección sensible de ZEA en alimentos infantiles. Estos resultados fueron refrendados mediante el análisis de dos muestras reales y representativas, correspondientes a alimentos infantiles que contienen cereales y de diversa complejidad, tales como un batido de leche y una papilla en polvo.

A partir de esta exitosa metodología de partida y teniendo en cuenta las extraordinarias propiedades que ofrece la tecnología de biosensores, la siguiente aproximación se centró en el desarrollo de un inmunosensor electroquímico soportado sobre partículas magnéticas y que hace uso de electrodos serigrafiados de carbono desechables. La transferencia de la metodología anteriormente desarrollada sobre la superficie de electrodos serigrafiados de carbono, permite la posibilidad de producir un sistema desechable y con la capacidad de llevar a cabo análisis *in situ* de forma sencilla. En este caso y una vez finalizada la reacción de bioreconocimiento, las partículas magnéticas sobre cuya superficie ha tenido lugar la formación de los correspondientes inmunocomplejos, son confinadas gracias a la ayuda de un imán, en la superficie de un electrodo serigrafiado de carbono para su detección electroquímica. Además de los beneficios inherentes a esta tecnología, se hace preciso destacar unos excelentes LOD y exactitud, similares a los obtenidos en la estrategia inicial. Adicionalmente, y con fines de simplificar el tedioso protocolo de calibración que exige el trazado completo de la función logística log-log*it* de cuatro parámetros utilizada de forma habitual, se ha propuesto un protocolo de calibración simplificada. En este sentido, se puede llevar a cabo de manera secuencial la calibración y el análisis de ZEA, utilizando para ello el mismo electrodo serigrafiado de carbono. Se obtuvieron excelentes características analíticas en términos de exactitud y precisión, con un bajo error sistemático (inferior al 4%) y una excelente reproducibilidad (RSD = 6%). Esta estrategia refuerza los méritos analíticos del inmunosensor electroquímico convirtiéndole en una herramienta analítica desechable muy prometedora para realizar análisis *in situ* en muestras de alimentos.

La segunda estrategia miniaturizada que se ha explorado en esta Tesis, se basa en la combinación del inmunoanálisis electroquímico con plataformas microfluídicas. Esta estrategia hace uso de los beneficios aportados por los sistemas microfluídicos, tales como tiempos de análisis rápidos, la reducción del consumo de muestras y/o reactivos, la

posibilidad de llevar a cabo análisis paralelos y de forma simultánea en un único dispositivo, a la vez de convertirse en una plataforma ideal para los microsistemas de análisis de inyección en flujo, ya que permite un control riguroso de los fluidos y por una tanto una mayor precisión en las distintas operaciones analíticas que configuran un microsistema de análisis total.

En un paso previo hacia la integración total del inmunoensayo electroquímico en una plataforma microfluídica, se ha desarrollado una estrategia inicial que permite la monitorización electroquímica *"on-chip"* del producto generado por la reacción enzimática correspondiente al método ELISA. Una vez tienen lugar las interacciones propias a un ELISA en pocillos individuales y "*off-chip*", el producto enzimático es inyectado electrocinéticamente y conducido para su detección electroquímica "*on-chip*". Con fines de conseguir una simplificación metodológica, se ha propuesto la utilización de los dos reservorios del chip para poder integrar las señales correspondientes a la calibración metodológica y el inmunoanálisis propiamente dicho, y así obtener la determinación de ZEA. A través de esta estrategia se han determinado fácilmente concentraciones inferiores a 1 ppb, lo que permite el control de ZEA muy por debajo de los límites máximos permitidos por la legislación para alimentos infantiles.

Finalmente, y atendiendo a lo que podría ser considerado el objetivo último de esta Tesis, se llevó a cabo la integración total de los diferentes pasos correspondientes al bioreconocimiento y a la detección electroquímica en un único y sencillo dispositivo microfluídico. Los beneficios derivados del uso de partículas magnéticas (aumento del área superficial, fácil manipulación para ejecutar las distintas etapas), así como de los microcanales (reducción del volumen de muestras y reactivos, disminución de las distancias de difusión para las interacciones biológicas) y de la detección electroquímica, provocan una gran mejora en la eficiencia del inmunoensayo.

Esta estrategia implica un uso elegante y creativo de un sencillo microchip con configuración de doble T, que permite realizar de forma secuencial tanto la inmunoreacción como la reacción enzimática dentro del propio dispositivo. Todo ello tiene lugar mediante la aplicación de una combinación de campos eléctricos a los diferentes

reservorios para conducir los fluidos a las distintas zonas (canales) del chip y realizar las correspondientes reacciones.

Bajo estas condiciones de funcionamiento, el sistema ha demostrado su capacidad para determinar la micotoxina ZEA reduciendo el tiempo total de análisis a 15 minutos, evitando los pasos manuales en el procedimiento y utilizando sólo unos pocos nanolitros de reactivos. El límite de detección obtenido ha sido de 0,4 ppb, con un bajo error sistemático del 2% en el análisis de un material de referencia certificado de ZEA en maíz, así como unas excelentes recuperaciones comprendidas entre el 103 y 101% para el análisis de muestras reales de naturaleza sólida y líquida respectivamente.

I INTRODUCCIÓN

En este primer capítulo se detallan la metodología y la problemática analítica sobre la que se desarrolla la presente Tesis doctoral.

En este sentido, se presenta una visión general sobre los Métodos Inmunoanalíticos y su utilización en la determinación y el control de la micotoxina Zearalenona.

I.1. GENERALIDADES SOBRE INMUNOANÁLISIS

I.1.1 Fundamento de los métodos inmunoanalíticos

Los métodos inmunoanalíticos se basan en la habilidad que presentan los anticuerpos (Ab) para formar complejos con sus correspondientes antígenos (Ag). Esta interacción se caracteriza por la elevada especificidad en el reconocimiento a nivel molecular de los antígenos por parte de los anticuerpos y por la elevada constante de afinidad de dicha interacción. Ambas características dotan a los métodos inmunoanalíticos de una elevada selectividad y sensibilidad, convirtiéndolos en una herramienta fundamental en diversos campos de aplicación como el análisis clínico y bioquímico, el análisis de alimentos y el medioambiental.

Por otra parte, a estas características analíticas, se le suma la facilidad y capacidad de las técnicas actuales de diseño y obtención de anticuerpos, lo que implica que puedan producirse anticuerpos para prácticamente cualquier tipo de macromolécula de interés[1,2]. Esto implica que los métodos inmunoanalíticos puedan ser empleados en la determinación de un amplio grupo de sustancias. Además, es importante destacar que gracias a las peculiaridades de los métodos inmunanalíticos anteriormente citadas, su utilización conlleva una reducción drástica -cuando no plena- del tratamiento de muestra y suponen una estrategia muy valiosa para el Químico Analítico a la hora de abordar problemáticas de muy diferente naturaleza. Dentro de estos métodos, la molécula de anticuerpo se erige como una herramienta analítica que reconoce de forma específica la sustancia que se desea analizar, y son por tanto, sus características peculiares las que determinan la naturaleza de esta metodología.

Los anticuerpos son proteínas especializadas, pertenecientes al grupo de las inmunoglobulinas, producidas por los linfocitos B. Forman parte del sistema inmunitario y se producen en respuesta a la presencia de moléculas extrañas en el organismo. Su unión con un antígeno pone en marcha distintos mecanismos biológicos, que actúan con el objetivo de eliminar los posibles efectos adversos producidos por la presencia de dichas moléculas[3].

La estructura básica de los anticuerpos consiste en cuatro cadenas polipeptídicas (150 kDa), unidas entre sí mediante puentes disulfuro. Dos cadenas se denominan ligeras (2 x 25 kDa) y las otras dos pesadas (2 x 50 kDa). Como en toda proteína, en cada cadena se distingue un extremo amino terminal (N-terminal) y un extremo carboxilo terminal (C-terminal). La fracción N-terminal se caracteriza por regiones variables (V), tanto en las cadenas pesadas (V_H), como en las cadenas ligeras (V_L), cuya secuencia de aminoácidos es propia de cada anticuerpo y contribuye en la especificidad de la unión antígeno-anticuerpo. El resto de la molécula posee una estructura constante (C_H), con la misma secuencia de aminoácidos en todos los anticuerpos, que es característica de cada una de las clases de inmunoglobulinas existentes[4, 5].

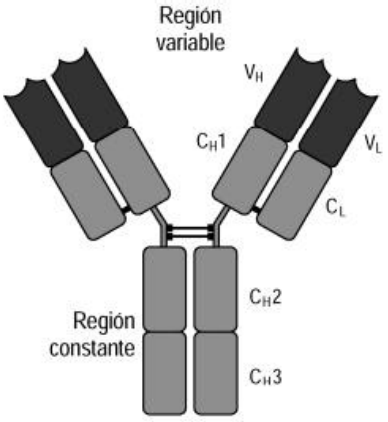

Figura 1. Esquema ilustrativo de una molécula de anticuerpo

Basándose en su actividad fisiológica, en sus características físico-químicas y en pequeñas variaciones de la secuencia de aminoácidos de las cadenas pesadas se pueden distinguir cinco clases principales de inmunoglobulinas. Cada clase se designa con una letra y se dividen en: IgG, IgA, IgM, IgD y IgE[6], siendo las IgGs las más utilizadas en inmunoensayos, debido a su papel predominante en el desarrollo de la respuesta inmune del organismo así como por la elevada afinidad que presentan frente a elementos antigénicos.

La región variable de los anticuerpos es la zona responsable de la interacción con el antígeno. Este hecho justifica que no siempre sea necesario emplear la molécula del anticuerpo completa, por lo que existen determinadas estrategias tanto enzimáticas como

basadas en técnicas de ingeniería genética que nos conducen a la obtención de las regiones útiles del reactivo.

En el caso clásico de la acción llevada a cabo por parte de enzimas proteolíticas, el tratamiento con papaína produce tres fragmentos, dos fragmentos idénticos correspondientes al extremo N-terminal (regiones variables) con capacidad para unir el antígeno, denominados Fab (del inglés, *fragment antibody binding*) y un fragmento Fc (del inglés, *crystallizable fragment*) correspondiente al extremo C-terminal, constituido por la región constante del anticuerpo. El tratamiento con pepsina produce un fragmento $F(ab)_2$, que consta de dos fragmentos Fab unidos covalentemente, y el resto de la molécula se degrada en fragmentos más pequeños denominados pFc[7].

Actualmente, mediante técnicas de ingeniería genética se pueden obtener fragmentos Fab con las características deseadas. Estos fragmentos se denominan scFv (del inglés, *single-chain fragment variable*) y comprenden los dominios variables de la cadena pesada (V_H) y de la cadena ligera (V_L) del anticuerpo, unidos entre sí mediante un péptido sintético. Mediante estas técnicas se pueden obtener fragmentos más pequeños que sólo contienen la cadena pesada o la ligera, y que mantienen en mayor o menor grado la afinidad por el antígeno.

Del mismo modo, la detección de cada antígeno requiere la producción de un anticuerpo específico, su aislamiento y en la mayoría de los casos, su purificación.

La producción de anticuerpos puede ser provocada en un animal al ser inyectado con una sustancia de alto peso molecular que sea reconocida como un compuesto extraño para el organismo. De esta forma el animal desarrolla su respuesta inmunológica, dando lugar a los anticuerpos deseados. Las características de los anticuerpos generados estarán relacionadas con las formas de producción. En este sentido, se puede distinguir entre anticuerpos policlonales y anticuerpos monoclonales.

Los anticuerpos policlonales son poblaciones complejas formadas por distintos tipos de anticuerpos. Estos se producen a partir del suero obtenido de un animal en el cual se ha inyectado previamente un inmunógeno. Por el contrario, los anticuerpos monoclonales son moléculas idénticas que poseen la misma especificidad. El primer método para la

producción de anticuerpos monoclonales fue descrito por Kolhler y Milstein en 1975, y se basa en el hecho de que cada linfocito B (células del sistema inmunitario), produce un tipo de anticuerpo específico. Así, aislando una célula e inmortalizándola mediante fusión celular con una célula cancerígena de mieloma, se pueden obtener anticuerpos idénticos de forma ilimitada.

En la actualidad, gracias a los avances recientes en técnicas de ingeniería genética, es posible la producción de anticuerpos en ausencia de la inmunización del animal. Es la denominada tecnología de los anticuerpos recombinantes, que permite la producción, identificación y conjugación de los fragmentos de la molécula de anticuerpo correspondientes a la zona de reconocimiento. La mayor ventaja de esta tecnología es la posibilidad de preparar anticuerpos análogos a los ya existentes, así como la creación de nuevos anticuerpos específicos para varios antígenos[8].

Por otra parte, se denomina antígeno a cualquier sustancia extraña a la que puede unirse un anticuerpo, si además es capaz de desencadenar una respuesta inmunitaria en el interior del organismo, se denomina inmunógeno[9].

No todas las moléculas son capaces de generar anticuerpos, sino que dicha capacidad está relacionada con el tamaño molecular. Los inmunógenos son sustancias de alto peso molecular que son reconocidas por el organismo como sustancias extrañas, desencadenando en el mismo una respuesta inmune con la correspondiente producción de anticuerpos. Por el contrario, los haptenos son sustancias de bajo peso molecular, que aunque son reconocidos por los anticuerpos, por sí solas no producen respuesta inmune en un organismo, por lo que deben conjugarse previamente con una molécula de naturaleza proteica para así producir la respuesta inmunológica.

Los anticuerpos reconocen a los antígenos, de modo que se produce la unión entre los determinantes antigénicos (epítopos), que consisten en una zona flexible con grupos químicos que favorecen la interacción, y las regiones variables de los anticuerpos (paratopos), produciéndose así la formación del complejo antígeno-anticuerpo[3].

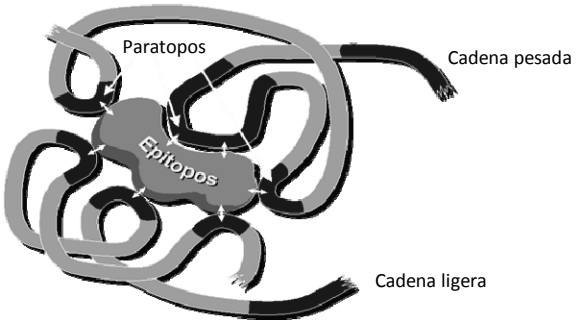

Figura 2. Esquema ilustrativo de la interacción antígeno-anticuerpo.

Para que la reacción antígeno-anticuerpo tenga lugar, ambas moléculas deben estar próximas y adecuadamente orientadas para que las interacciones (entre los epítopos y los paratopos) sean efectivas.

La elevada afinidad entre el antígeno y el anticuerpo es debida a fuerzas de naturaleza no covalente, tales como puentes de hidrógeno, interacciones hidrofóbicas y fuerzas de Van der Waals, por lo que es una unión reversible.

La formación del complejo antígeno-anticuerpo se puede dividir en dos partes: el reconocimiento y la interacción. Se puede considerar que, para el reconocimiento y la formación inicial del complejo antígeno-anticuerpo, las interacciones electrostáticas y polares de relativo largo alcance juegan el papel más importante, mientras que los enlaces por puente de hidrógeno son las interacciones dominantes en la formación del complejo. La suma de estas fuerzas de atracción y repulsión se conoce como afinidad del anticuerpo por el antígeno[6]. Por otra parte, la avidez es la medida de la estabilidad total del complejo, determinado por la afinidad del anticuerpo por el epítopo, el número de sitios de unión por la molécula de anticuerpo y la disposición geométrica de los componentes que interaccionan. La avidez describe todos los factores que rodean a la reacción de interacción, los cuales determinan el éxito de las técnicas inmunoquímicas[10]. Debido a que en muchos casos los antígenos son multivalentes y los anticuerpos también, esta multivalencia tiende a aumentar la afinidad funcional o avidez [11].

La unión antígeno-anticuerpo es un equilibrio dinámico, representado por la siguiente ecuación:

$$Ab + Ag \longleftrightarrow Ab\text{-}Ag$$

Siendo K = [Ab-Ag] / [Ab][Ag], la constante de afinidad del complejo; [Ag-Ab] la concentración del complejo antígeno-anticuerpo; [Ag] la concentración de antígeno libre y [Ab] es la concentración de anticuerpo libre. Esta ecuación nos indica que para unas condiciones determinadas de temperatura, pH y fuerza iónica, la relación de concentraciones entre el complejo y los reactivos en equilibrio es siempre constante. La constante de afinidad (K) constituye una medida cuantitativa sobre la fuerza con que un determinado anticuerpo reacciona con su antígeno específico. Los valores habituales de K comprenden desde valores bajos como $5x10^4$ hasta valores elevados del orden de 10^{12} $L{\cdot}mol^{-1}$.

Como se ha mencionado anteriormente, la interacción es debida a fuerzas no covalentes, por lo que es reversible y en consecuencia se ve afectada por factores como la fuerza iónica, las concentraciones de antígeno y anticuerpo, la temperatura y el pH. Este hecho es especialmente relevante en ciertas metodologías inmunoanalíticas, donde tras la correspondiente interacción y con objeto de llevar a cabo un nuevo análisis, se hace necesario regenerar la molécula de anticuerpo. A modo de ejemplo, las interacciones electrostáticas son muy dependientes del pH (aunque no se ha determinado un pH óptimo, se supone que se aproxima al pH fisiológico) y de la fuerza iónica (cuando la concentración salina del medio disminuye, la velocidad de reacción aumenta). A su vez, la temperatura tiene efectos inversamente proporcionales con la constante de equilibrio y la velocidad de reacción (la mayoría de los anticuerpos presentan un rango térmico específico). También hay que indicar que los disolventes orgánicos pueden debilitar las interacciones antígeno-anticuerpo.

I.1.2 Clasificación de los métodos inmunoanalíticos

Tal y como se ha comentado anteriormente, esta metodología viene determinada por la interacción antígeno-anticuerpo. Sin embargo, y a diferencia de otras reacciones de naturaleza biológica como la catálisis enzimática, en dicha interacción no se genera ninguna conversión química. Este hecho dificulta su detección y explica la existencia de múltiples estrategias de transducción que generan diferentes criterios y clasificaciones de los métodos inmunoanalíticos. De este modo, y aunque existen técnicas capaces de medir directamente la formación del complejo antígeno-anticuerpo (cambios másicos o en el índice de refracción), la mayor parte de los métodos inmunoanalíticos hacen uso de un marcador y se denominan **inmunoanálisis con marcador**. Este marcador desarrolla una señal medible y que puede relacionarse con el grado de interacción entre el antígeno y el anticuerpo. En función de la naturaleza del marcador aparecen distintas metodologías, pudiendo ser éste una molécula radioactiva, fluorescente o una enzima dando lugar al radioinmunoanálisis (RIA), fluoroinmunoanálisis (FIA) o quimioluminoinmunoanálisis (LIA) y enzimoinmunoanálisis (EIA)[12], respectivamente.

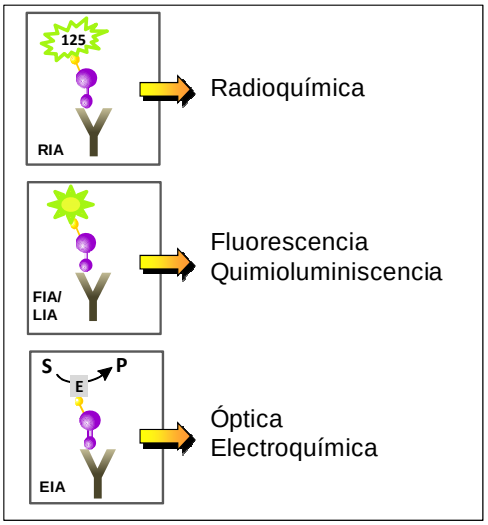

Figura 3. Tipos de inmunoensayo con marcador.

Asimismo, un criterio importante es aquel que nos permite establecer una clasificación basándose en el grado de ocupación de los sitios de unión del anticuerpo por el antígeno, dando lugar a los denominados **métodos no competitivos y competitivos**.

Los métodos competitivos determinan los sitios de unión del anticuerpo no ocupados por el antigeno. Tal y como se muestra en el esquema, en el ensayo competitivo el antígeno de la muestra (Ag) compite, con un derivado del antígeno marcado (Ag*), por un número limitado de sitios de unión del anticuerpo (Ab), que se encuentra en concentración limitante. Si la concentración de analito aumenta, el derivado marcado es desplazado, dando lugar así, a una menor señal.

$$Ag + Ag^* + Ab \underset{Kd}{\overset{Ka}{\rightleftharpoons}} Ag\text{-}Ab + Ag^*\text{-}Ab + Ag^* + Ag$$

Por el contrario, los métodos no competitivos miden directamente los sitios ocupados, por lo que la señal desarrollada por el marcador será directamente proporcional a la concentración de analito (antígeno) en la muestra.

Otro criterio ampliamente utilizado, clasifica los métodos inmunoanalíticos en **homogéneos y heterogéneos**. En los métodos homogéneos, tras la interacción antígeno-anticuerpo, no se precisa de la separación del marcador que ha quedado unido respecto del que se encuentra en disolución. En este caso, se produce una alteración en la señal que origina el marcador cuando forma parte del complejo antígeno-anticuerpo. Sin embargo, en los métodos heterogéneos, la señal no se modifica tras la formación del complejo y obliga a introducir un paso de separación para eliminar el inmunoreactivo sobrante. Los métodos heterogéneos presentan mejor sensibilidad, ya que no están expuestos al ruido de fondo, pero requieren que anticuerpo o antígeno sean inmovilizados sobre un soporte sólido.

De la amplia variedad de métodos inmunoanalíticos que se pueden encontrar en la bibliografía, destacan por su amplia utilización aquellos que utilizan una enzima como marcador, dando lugar al enzimoinmunoanálisis (EIA, *enzyme immunoassay*). A su vez y, dentro de estos, cabe resaltar la metodología ELISA (*Enzyme-Linked Immunosorbent Assay*) correspondiente a los métodos heterogéneos y, que por tanto, implican la inmovilización de alguna de las especies en un soporte sólido.

I.1.3 Enzimoinmunoanálisis: ELISA (*Enzyme-Linked ImmunoSorbent Assay*)

El empleo de enzimas como marcadores inmunoquímicos fue introducido por primera vez en 1971 como alternativa a los isótopos radiactivos[13]. Estos marcadores no producen una medida directa como ocurre con los isótopos radioactivos y los fluoróforos, pero permiten llevar a cabo la medida indirecta del grado de interacción antígeno-anticuerpo, a través de la adición de un substrato que reacciona con el enzima generando un producto medible.

Las enzimas son proteínas que reaccionan selectivamente con un analito o familia de analitos y que son capaces de catalizar una reacción química. Su empleo como marcadores aporta una serie de ventajas, entre las que cabe destacar su facilidad de uso, bajo coste, estabilidad en los intervalos de pH, fuerza iónica y temperatura en la que se realizan los ensayos, así como la posibilidad de utilizar diferentes substratos para los que presentan bajas constantes de Michaelis (Km) y que dan lugar a productos detectables por distintas técnicas. Sin embargo, la gran ventaja de utilizar las enzimas como marcadores y que han dado lugar a su amplia utilización, es el efecto de amplificación de la señal gracias a las elevadas constantes catalíticas (una molécula de enzima es capaz de generar un elevado número de moléculas de producto), lo que los convierte en métodos altamente sensibles. Estas características convierten a las enzimas en los marcadores más atractivos y empleados. Esto, sumado al gran poder discriminante característico de los anticuerpos, permite que estas técnicas sean capaces de cuantificar analitos (incluso a niveles de trazas) sin la necesidad de procedimientos de purificación y/o concentración de la muestra.

Algunas de las características que deben presentar las enzimas utilizadas como marcadores incluyen la posibilidad de llevar a cabo una fácil derivatización de los antígenos y/o anticuerpos, encontrarse en estado puro a un precio razonable y tener un substrato conveniente: cromogénico, fluorogénico o electroquímico. Las enzimas más representativas y utilizadas en enzimoinmunoensayo son la fosfatasa alcalina, la glucosa oxidasa y la peroxidasa de rábano picante (HRP, *Horseradish peroxidase*).

En este trabajo se ha utilizado la peroxidasa de rábano picante (HRP). Esta enzima en presencia del H_2O_2 cataliza la oxidación de un donador de electrones tal como la

hidroquinona (HQN) pasando a benzoquinona (BQN) y, que en nuestro caso, se ha detectado mediante su reducción electroquímica en la superficie del electrodo. Este proceso se repite cíclicamente mientras no exista limitación por la concentración de substrato, dando lugar al fenómeno de amplificación anteriormente comentado, y que finalmente, dependiendo de la metodología inmunoanalítica empleada, se relaciona con la cantidad de analito presente en la muestra.

Aunque el enzimoinmunoanálisis (EIA) engloba tanto a los métodos homogéneos, en los que la actividad enzimática se ve influenciada por la interacción antígeno-anticuerpo, siendo el EMIT (*Enzyme Multiplied Immunoassay Technique*) o Inmunoensayo enzimático multiplicado el más representativo, resulta preciso indicar que son los métodos heterogéneos ELISA, los más ampliamente utilizados. Esta es además la metodología empleada en el desarrollo de este trabajo de Tesis y será por tanto, descrita con mayor grado de detalle.

El análisis de inmunoadsorción ligado a enzimas o ELISA, hace uso de las elevadas constantes catalíticas de las enzimas anteriormente mencionadas produciendo una gran amplificación de la señal correspondiente a cada interacción antígeno-anticuerpo. Además y gracias a la posibilidad de utilizar substratos diferentes, su producto de reacción puede ser detectado por distintas técnicas, tanto ópticas como electroquímicas.

Debido a su carácter de método heterogéneo, una de las especies inmunológicas se encuentra inmovilizada en una fase sólida, tradicionalmente en los pocillos de una placa de microtitulación de poliestireno. Tras la formación del complejo antígeno-anticuerpo formado en la fase sólida, éste se separa de los reactivos libres en disolución, antes de proceder a la medición del grado de interacción a través de la reacción enzimática.

A su vez esta metodología puede presentarse en un formato competitivo o no competitivo. Las tres modalidades más utilizadas son las siguientes:

ELISA no competitivo

Los métodos no competitivos habitualmente se utilizan para la determinación de anticuerpos. Para ello, el antígeno en una primera fase es adsorbido en una superficie sólida, posteriormente se adiciona la muestra que contiene el anticuerpo (analito) y se deja reaccionar. Por último, la adición de un anticuerpo secundario marcado que reconoce de modo específico al primer anticuerpo permite cuantificar la cantidad de analito en la muestra. La señal desarrollada por el marcador será directamente proporcional a la concentración de analito (anticuerpo) en la muestra. La **Figura 4** muestra un esquema de este tipo de inmunoensayo.

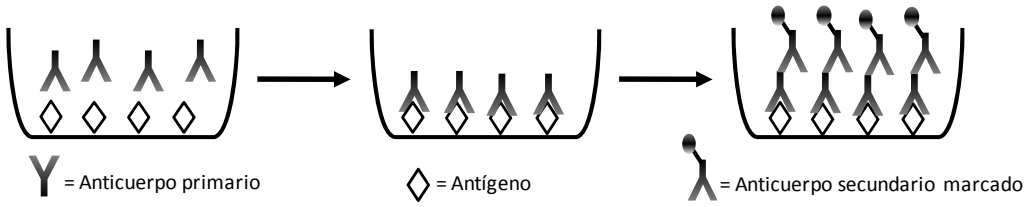

Figura 4. Esquema correspondiente a un inmunoensayo no competitivo.

ELISA no competitivo formato sándwich

En la mayor parte de los casos, el ELISA no competitivo suele realizarse en formato sándwich. En este formato se utilizan dos tipos de anticuerpos distintos, cada uno capaz de reconocer un determinante antigénico diferente del mismo antígeno, que debe ser polivalente. En la variedad más habitual, uno de los anticuerpos está inmovilizado en el soporte sólido (anticuerpo de captura) y el otro está conjugado con una enzima (anticuerpo de detección). Después de la incubación secuencial, el antígeno (analito) queda unido entre los dos anticuerpos. Los dos anticuerpos deben estar en exceso. El primero, para asegurar la adsorción completa del antígeno y el segundo, para asegurar la marcación completa del inmunocomplejo. En este caso, la concentración de analito es directamente proporcional a la cantidad de enzima unida al complejo. Los métodos no competitivos presentan ciertas ventajas, como límites de detección más bajos; sin embargo precisan de antígenos que presenten más de un determinante antigénico puesto que han de ser reconocidos por dos anticuerpos distintos. Debido a este motivo, es por lo que el método sándwich no se puede

aplicar, por lo general, a moléculas pequeñas. El esquema correspondiente a este tipo de inmunoensayo se muestra en la **Figura 5**.

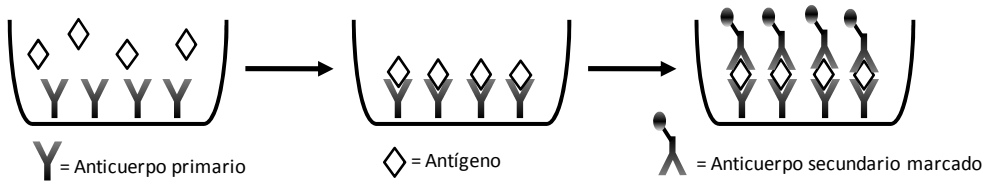

Figura 5. Esquema correspondiente a un inmunoensayo no competitivo en formato *sándwich*

ELISA competitivo

Este tipo de esquema de inmunoensayo, se utiliza para la detección y cuantificación de antígenos y se basa en la competición entre antígeno marcado y no marcado. Tal y como se ha recogido en la **Figura 6**, en el formato habitual, un anticuerpo no marcado es inmovilizado sobre un soporte sólido, ya sea directamente o bien mediante un segundo anticuerpo que reconoce al primero. A continuación, una cantidad conocida de un derivado del antígeno marcado es mezclada con la muestra problema y añadida al anticuerpo inmovilizado. El antígeno de la disolución problema compite con el derivado marcado por los sitios de unión del anticuerpo. Si la concentración de antígeno en la muestra es elevada, la cantidad de antígeno marcado unido al anticuerpo será baja, así como la señal obtenida tras la adición del substrato enzimático. Otra posibilidad es llevar a cabo la inmovilización del antígeno (o de un derivado) sobre el soporte sólido, añadiendo posteriormente la muestra y el anticuerpo marcado. El analito de la muestra y el inmovilizado compiten por los sitios de unión al anticuerpo. Al igual que en el caso anterior, al aumentar la concentración de analito en la muestra, la concentración de anticuerpo marcado unido al antígeno inmovilizado disminuye, dando lugar a una disminución de la señal tras la reacción enzimática.

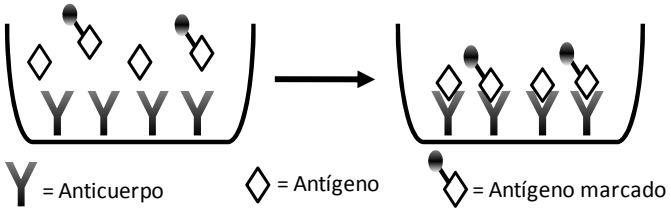

Figura 6. Esquema correspondiente a un inmunoensayo competitivo directo.

Aunque de forma tradicional la detección óptica ha sido la más ampliamente utilizada en esta metodología, resulta preciso indicar que en los últimos años y, fundamentalmente bajo el diseño de los denominados immunosensores, la detección electroquímica ha alcanzado un elevado protagonismo. Este hecho se fundamenta en las características interesantes de la detección electroquímica, tales como su miniaturización inherente, su independencia de la turbidez de la muestra, su bajo coste y su elevada sensibilidad. Asimismo, la instrumentación puede ser fácilmente miniaturizada, permitiendo la detección en volúmenes pequeños de muestra. Otra de las ventajas que ofrece la detección electroquímica es el elevado número de moléculas electroactivas que existen. La detección electroquímica se puede llevar a cabo mediante un marcaje directo, donde el antígeno (o anticuerpo) es marcado con una molécula electroactiva, o bien lo más habitual es utilizar un marcaje enzimático, donde el antígeno (o anticuerpo) es marcado con una enzima, cuyo producto de reacción puede ser detectado electroquímicamente. Los productos electroactivos generados se pueden medir mediante una serie de técnicas electroquímicas dentro de las cuales, las más comúnmente utilizadas son las técnicas amperométricas y las voltamperométricas. En las primeras se aplica un potencial constante sobre el electrodo de trabajo y se mide la intensidad de la corriente resultante que circula por el electrodo. Por su parte, en las técnicas voltamperométricas se produce la aplicación de un potencial variable sobre el electrodo de trabajo, seguida de la medida de la intensidad de la corriente resultante de la reacción electródica. En ambos casos, la intensidad medida será función del potencial aplicado y de la concentración de la especie electroactiva presente.

I.1.4 Estrategias de inmovilización en soportes sólidos en los métodos ELISA

Tal y como se ha mencionado anteriormente, esta metodología requiere de una etapa que permita separar los inmunoreactivos libres de los inmunocomplejos formados. Para este fin es necesario que una de las especies se encuentre inmovilizada en un soporte sólido. De forma habitual y, coincidiendo con la estrategia seguida en este trabajo de Tesis, suele ser el anticuerpo específico la especie a inmovilizar en un soporte sólido. En la **Figura 7** se presenta una clasificación de las técnicas habituales de inmovilización de anticuerpos[14-16]. La

estrategia de inmovilización ha de aportar estabilidad a la biomolécula y ha de asegurar su actividad biológica.

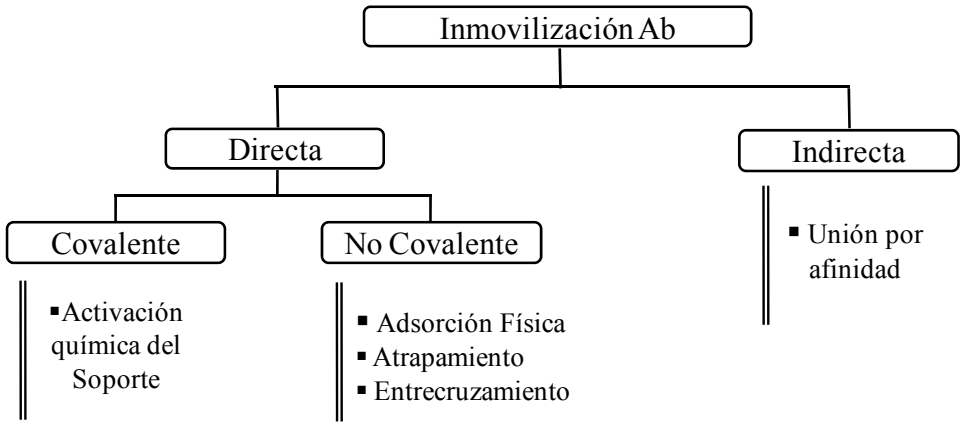

Figura 7. Estrategias de inmovilización de anticuerpos.

El objetivo principal para una adecuada inmovilización es que el anticuerpo se una a la superficie a través de su región Fc, de forma que, la región Fab quede expuesta al medio, minimizando los impedimentos estéricos y siendo capaz de reconocer el antígeno sin ningún obstáculo. Sin embargo, no existe ninguna estrategia ideal para inmovilizar el anticuerpo, de forma que, dependiendo del uso que le queramos dar a nuestro anticuerpo inmovilizado, deberíamos escoger el método de inmovilización que más se adapte a nuestras necesidades[15].

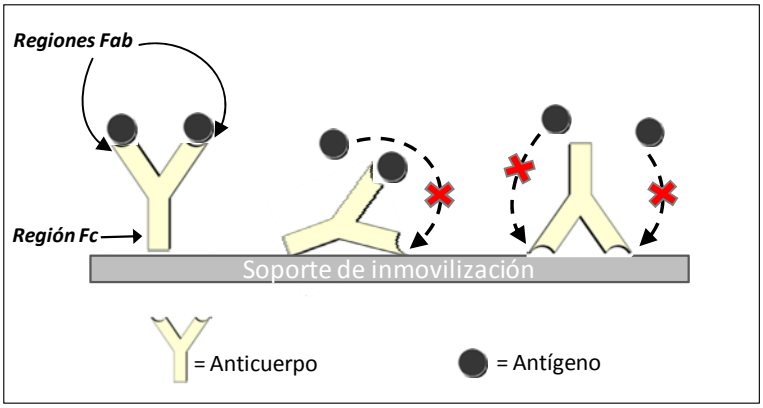

Figura 8. Esquema ilustrativo sobre diferentes disposiciones que puede adoptar un anticuerpo durante el proceso de inmovilización sobre un soporte sólido.

Otro aspecto a tener en cuenta, es evitar la posible adsorción inespecífica (unión no deseada de proteínas), ya que puede reducir la sensibilidad del sistema. Normalmente, una vez que

se ha unido el anticuerpo al soporte de inmovilización, es necesario introducir alguna etapa de bloqueo, para conseguir tapizar el soporte utilizado y evitar que se unan otras proteínas presentes en la disolución[17]. De esta forma, nos aseguraremos que el analito está reaccionando sólo con el anticuerpo y que la unión se está produciendo de forma específica. A continuación, se comentarán las diferentes técnicas de inmovilización.

I.1.4.1 Técnicas directas de inmovilización

La técnica más utilizada implica la simple **adsorción física** de la molécula de anticuerpo sobre una superficie de poliestireno o cualquier otro material polimérico con capacidad de unir proteínas[15]. Esta unión tiene lugar a través de interacciones electrostáticas, hidrofóbicas, puentes de hidrógeno y fuerzas de Van der Waals[14-15]. Sin embargo, este tipo de inmovilización supone aleatoriedad en la posición del anticuerpo (baja reproducibilidad), pudiéndose bloquear los sitios de unión de muchas de estas moléculas, además de su posible desnaturalización y cierta facilidad para su desorción[14-15].

Otra técnica directa es el **atrapamiento**, que consiste en la retención física de uno de los inmunoreactivos en las cavidades de una matriz sólida porosa. Generalmente se utilizan matrices poliméricas (polímeros inertes como la poliacrilamida, matrices de polisulfona…) o geles. Esta técnica requiere un control riguroso de las condiciones de polimerización, así como la comprobación de que la naturaleza química del proceso no altera los grupos reactivos de la proteína.

Un ejemplo importante es la utilización de polímeros electroactivos como el polipirrol o la polianilina para llevar a cabo el atrapamiento directamente sobre la superficie de un transductor. Estos polímeros mejoran la transferencia electrónica entre el centro activo de la enzima (utilizada como marcador) y la superficie del transductor. Del mismo modo, la utilización de biocompositos conductores que integran el material biológico, el mediador, el transductor y una matriz que permita el atrapamiento, aportan una serie de ventajas para el desarrollo de inmunosensores.

Otra forma de atrapamiento es el encapsulamiento o la inclusión en membranas semipermeables (permiten el paso de determinadas moléculas). Dentro de ellos, los soportes

sol-gel han emergido como una plataforma prometedora basada en la preparación de redes tridimensionales adecuadas para la inmovilización de una gran variedad de moléculas biológicas, tales como enzimas y anticuerpos.

Por su parte, el **entrecruzamiento** (*cross-linking)*, supone la unión irreversible de los elementos de reconocimiento entre sí mediante agentes bifuncionales (dialdehídos, diiminoésteres…) siendo los más utilizados el glutaraldehído o la polietilenimina. Dichos reactivos tienen dos grupos carbonilo terminales que pueden reaccionar con grupos amino de las proteínas formando agregados insolubles. La principal limitación de esta estrategia es que las capas formadas suelen ser gruesas y dificultan la difusión de especies a través de las mismas.

Por último, otras técnicas emplean la inmovilización mediante una **unión covalente**. Esta unión es irreversible, basándose en la reacción entre grupos reactivos de la superficie sólida utilizada y grupos reactivos de los inmunoreactivos (no importantes para la actividad biológica). Esta estrategia es la que asegura una mayor estabilidad del material biológico durante un largo tiempo, aunque no su correcta orientación, tratándose de procesos complejos y laboriosos[15].

Una de las técnicas más utilizadas es la activación de grupos carboxilos mediante la utilización de la carbodiimida y N-hidroxisuccinimida. Estos reactivos forman ésteres reactivos que permiten activar los carboxilos del soporte de inmovilización (sin alterar la actividad de anticuerpo) o bien activar los carboxilos del anticuerpo para que reaccionen con soportes aminados. Un inconveniente de este tipo de inmovilización es la posible desorción del anticuerpo debido a reacciones indeseadas.

Asimismo, los enlaces covalentes formados entre mercapto derivados y las superficies de oro presentan una gran afinidad. Mediante la utilización de reactivos bifuncionales que presenten un grupo mercapto-, sulfuro o disulfuro en un extremo y el grupo funcional requerido en el otro extremo, puede inmovilizarse el inmunoreactivo deseado. La inmovilización de estas moléculas da lugar a estructuras ordenadas en forma de monocapas autoensambladas (SAMs, *self-assembled monolayers*). Este tipo de inmovilización permite

que exista un transporte de materia rápido del analito hacia la fase sensora, disminuyendo el tiempo de respuesta. Además mejoran el límite de detección, posibilitan la regeneración del sensor y evitan la adsorción inespecífica.

I.1.4.2 Técnicas indirectas de inmovilización

Otra alternativa más interesante hace uso de **interacciones biológicas** que presentan elevadas constantes de asociación, como el sistema avidina-biotina o las proteínas A y G. Este tipo de interacciones pueden evitar los posibles problemas de aleatoriedad en la inmovilización, así como la posible desnaturalización de la molécula de anticuerpo que puede ocurrir en otras modalidades de inmovilización. A continuación se detallan las interacciones biológicas más empleadas:

Unión biotina-avidina

La avidina es una glicoproteína tetramérica (67000D) que presenta cuatro lugares de unión por los cuales se puede unir a la biotina y conjugarse con diversos marcadores. Del mismo modo, la estreptavidina es una glicoproteína tetramérica de 60000 D, presente en la bacteria *Streptomyces avidinii* y que tiene una afinidad por la biotina comparable a la avidina; la diferencia es que no posee cadenas laterales de carbohidratos y evita reacciones no específicas.

Por otra parte, la biotina es la denominada vitamina H (C_{10} H_{16} N_2 O_3S), de bajo peso molecular (244 D) tiene una elevada afinidad (constante de asociación 10^{15} M^{-1}) por la avidina o la estreptavidina. Esta afinidad (biotina-(estrept-)avidina) es superior a la mostrada por la unión anticuerpo-antígeno[16,18,19]. Esta unión biotina-(estrept-)avidina posee una elevada afinidad y estabilidad, además preserva con más eficacia la función biológica de las moléculas inmovilizadas con respecto a otras técnicas utilizadas. A su vez, un gran número de moléculas pueden ser modificadas con biotina sin alterar su función biológica.

Proteínas A y G

Son proteínas monoméricas capaces de unirse a las regiones constantes (Fc) de los anticuerpos, permitiendo así la inmovilización orientada de los mismos. De este modo las regiones Fab quedan accesibles, permitiendo su unión con las moléculas de antígeno[16].

La proteína A, es una proteína monomérica de 46000 Daltons, aislada de la pared celular de *Staphilococcus aureos*. Tiene una elevada afinidad por las inmunoglobulinas de mamíferos, especialmente por las IgG. La unión entre la proteína A y la región Fc del anticuerpo, implica la formación de una serie de enlaces no covalentes, como puentes de hidrógeno, interacciones hidrofóbicas y electrostáticas y fuerzas de Van der Waals.

La proteína G, es una proteína monomérica de entre 30000 y 35000 Daltons, aislada de la pared celular bacteriana del *Streptococcus.* Su utilización favorece una mejor sensibilidad y reproducibilidad del ensayo. La proteína G difiere de la A en su capacidad de reconocimiento, siendo capaz de reconocer a las cuatro clases de IgG[16].

I.1.4.3 Soportes de inmovilización en los métodos ELISA

En el apartado anterior, se han estudiado los distintos mecanismos por los que se puede llevar a cabo la inmovilización de los reactivos inmunológicos, especialmente los anticuerpos. A continuación, se expondrá una breve perspectiva de los posibles soportes para dicha inmovilización. A menudo, la elección del soporte conlleva la utilización de determinadas estrategias de inmovilización e incluso el diseño de la metodología.

De entre los distintos soportes utilizados, se comentarán, dada su especial relevancia, las placas de microtitulación, las micropartículas[20-27], las membranas y aquellos soportes que actuarán no sólo como superficie de inmovilización sino también como transductores de la reacción bioquímica en una respuesta medible. Este último grupo, de especial importancia ha dado lugar a los denominados inmunosensores, de los cuales se hará especial mención aunque centrándose en los de naturaleza electroquímica.

Las **placas de microtitulación** son el soporte sólido más empleado. Se trata de placas planas con múltiples "pocillos" (normalmente 96) que se utilizan como tubos de ensayo. Se

fabrican en varios materiales, aunque el más habitual es el poliestireno gracias a la facilidad para unir proteínas y a su transparencia, lo que le ha permitido ser utilizado para detección óptica. Los resultados obtenidos pueden leerse de forma automática mediante la utilización de lectores espectrofotométricos de placas. El inmunoanálisis en placa ha demostrado ser de una gran utilidad. Sin embargo, presenta ciertos inconvenientes tales como baja reproducibilidad y un elevado tiempo de análisis en etapas tales como los lavados y las incubaciones. Una de las etapas más lentas dentro de este contexto es la difusión de los componentes de la muestra hacia la superficie de las paredes de poliestireno. Asimismo, es necesaria una etapa de bloqueo con el fin de tapizar la superficie de la placa y evitar así, adsorciones inespecíficas indeseadas. Sin embargo, sigue siendo una de las técnicas más utilizadas en análisis de rutina en laboratorios debido al gran número de muestras que se pueden procesar de forma rápida y simultánea. Actualmente se están utilizando placas con mayor capacidad de 384 y 1536 pocillos, idóneos para sistemas de *screening*.

Asimismo, las **micropartículas** son esferas poliméricas que resultan de gran utilidad como soporte de inmovilización de anticuerpos, ya que favorecen la reacción inmunológica debido al aumento del área superficial, y a que mejoran la cinética de reacción al encontrarse las partículas en suspensión. Como resultado global mejora también la sensibilidad de ensayo, debido a un aumento en la eficacia de las interacciones entre reactivos y muestra. A estas micropartículas se les pueden acoplar ligandos o grupos funcionales que les permiten llevar a cabo los mecanismos de inmovilización de biomoléculas (anticuerpos, antígenos, enzimas…) comentados en el apartado anterior (unión covalente, por afinidad). A su vez, existen diversos tipos de materiales para la fabricación de estas partículas tales como poliestireno, agarosa, vidrio de poro controlado.

Del mismo modo, las **micropartículas magnéticas** son esferas constituidas por un núcleo magnético recubierto de un polímero inerte. Estas partículas son paramagnéticas, por lo que sólo muestran comportamiento magnético en presencia de un campo magnético. A su vez, la superficie polimérica puede ser funcionalizada por grupos químicos o directamente por moléculas biológicas, permitiendo así el enlace con diferentes tipos de biomoléculas (proteínas, anticuerpos o ADN). Al igual que las micropartículas poliméricas, su utilización

como soporte sólido para inmovilizar los inmunoreactivos favorece la reacción inmunológica. Asimismo, el hecho de que presenten un núcleo magnético posibilita su manipulación y facilita la realización de etapas de separación, ya que éstas quedarán retenidas en el fondo del vial al aplicar un campo magnético. Otras ventajas que presentan son su capacidad de reutilización, su fácil dispersión en disoluciones acuosas y la escasa formación de agregados.

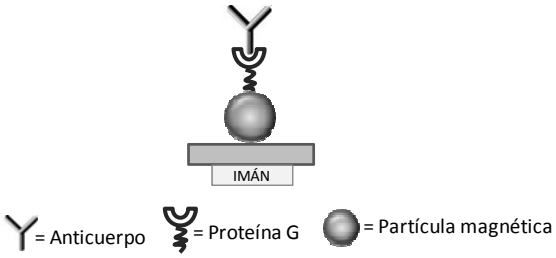

Figura 9. Partícula magnética recubierta con proteína G para la inmovilización orientada de anticuerpos.

En el caso de la inmovilización sobre **membranas**, el inmunoreactivo queda retenido entre el transductor y una membrana semipermeable que permite que las especies que intervienen en la reacción difundan a través de la misma. Estas membranas, debido a su elevada área superficial, producen un elevado rendimiento del proceso de inmovilización. Sin embargo, este tipo de inmovilización lleva asociado un alto grado de irreproducibilidad. Este inconveniente se puede solventar mediante la utilización de compuestos, como las proteínas A o G, que posibilitan una inmovilización orientada de los anticuerpos.

Con respecto a la inmovilización directa sobre la superficie de un **transductor**, que da lugar a los inmunosensores, se engloban aquellos inmunoensayos en los cuales la molécula de anticuerpo se inmoviliza directamente sobre la superficie de un transductor físico-químico que permite transformar la interacción biológica en una señal cuantificable, ya sea óptica, eléctrica, másica o térmica. La proximidad entre el material de bio-reconocimiento y el transductor, permite detectar los cambios debidos a la interacción entre el antígeno y el anticuerpo de forma más sensible. Estos dispositivos han aparecido como resultado a la creciente demanda de la sociedad actual de llevar a cabo análisis muy sensibles en campos cada vez más diversos, fruto de la combinación de las características de los sensores

químicos, con la gran especificidad de los análisis biológicos. De esta manera, surgieron los biosensores que engloban un grupo más amplio de dispositivos donde el elemento de reconocimiento puede ser enzimas, microorganismos, tejidos, anticuerpos, ácidos nucleicos, receptores, y que aparecieron como un método alternativo a las metodologías tradicionales de análisis, debido a la simplificación y abaratamiento, consiguiendo igualmente una elevada especificidad, sensibilidad y reproducibilidad. En la **Figura 10** se ilustra un esquema general de un biosensor.

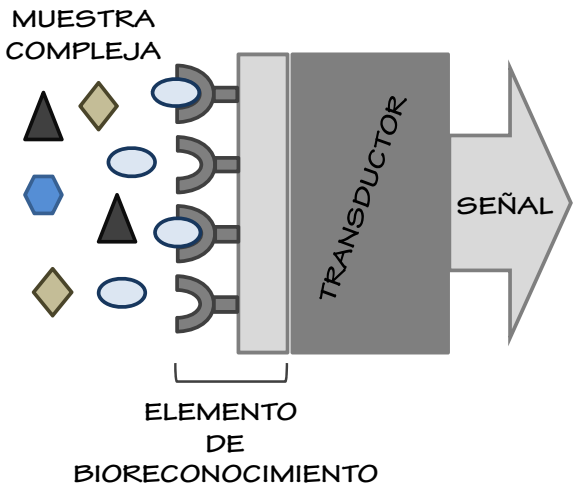

Figura 10. Esquema general de un biosensor.

Se puede establecer una clasificación en función de la naturaleza del sistema de transducción. Este puede ser electroquímico, óptico, piezoeléctrico, termométrico y nanomecánico.

Los **transductores electroquímicos** son aquellos que transforman la señal producida por la interacción antígeno-anticuerpo en una señal eléctrica. Existen cuatro tipos de transductores electroquímicos que son conductimétricos, potenciométricos, amperométricos e impedimétricos, dependiendo de que la medida se base en cambios producidos en la conductividad, el potencial, la corriente o la impedancia, respectivamente.

A su vez, los **transductores ópticos** se basan en los cambios que se producen en las propiedades de la radiación electromagnética (absorción, fluorescencia, luminiscencia, dispersión o índice de refracción) producidas como consecuencia de la interacción

antígeno-anticuerpo. Es preciso destacar la resonancia de plasmón superficial (SPR) que permite detectar de forma muy sensible la interacción antígeno-anticuerpo sin necesidad de marcador.

Los **transductores piezoeléctricos** permiten la detección directa de la interacción antígeno-anticuerpo sin necesidad de utilizar marcador, monitorizando cambios directos de masa debidos a la formación del complejo antígeno-anticuerpo. Los materiales piezoeléctricos son aquellos que entran en resonancia por la aplicación de un campo eléctrico externo. La frecuencia de oscilación de estos cristales, viene determinada por su masa, la cual varía cuando se produce la interacción entre el antígeno y el anticuerpo. Por tanto, la señal medida es la variación en la frecuencia de oscilación antes y después de que se produzca la interacción.

Los **transductores termométricos** se basan en la detección del calor generado en las reacciones enzimáticas exotérmicas, que se puede relacionar con la concentración de analito.

Por su parte, en los **transductores nanomecánicos** el anticuerpo se inmoviliza sobre la superficie de una micropalanca de silicio, que se sumerge en una muestra líquida. La interacción antígeno-anticuerpo produce un cambio diferencial en la tensión superficial del líquido y la micropalanca sufre una respuesta de tipo nanomecánico que consiste en un cambio de la frecuencia de resonancia.

I.2 GENERALIDADES SOBRE MICOTOXINAS

Las micotoxinas son sustancias naturales de bajo peso molecular producidas por hongos como metabolitos secundarios. Estos metabolitos constituyen un grupo muy heterogéneo, desde el punto de vista de su toxicidad y estructura química, que causan enfermedades y la muerte en humanos y otros vertebrados. Estos compuestos producidos como consecuencia del metabolismo secundario de hongos filamentosos, pueden aparecer en alimentos que han sido sometidos a ciertas condiciones medioambientales (elevada humedad y/o temperatura) que favorecen la infección del alimento por una determinada especie fúngica, durante la cosecha o el almacenamiento de los mismos. Las micotoxinas suelen aparecer en diversos tipos de productos vegetales, fundamentalmente, los cereales. Por ello, pueden estar presentes tanto en materias primas, como en productos derivados de alimentos esenciales de la dieta.

El término micotoxina se acuñó en 1960 a raíz del descubrimiento de las aflatoxinas, como consecuencia de una crisis veterinaria en Inglaterra conocida como "enfermedad X de los pavos" en la que murieron aproximadamente 100.000 pavos[33]. Finalmente, esta enfermedad se asoció a la contaminación de los cacahuetes utilizados como alimento de los pavos con metabolitos secundarios de *Aspergillus flavus* (aflatoxinas). Con el aislamiento de las aflatoxinas, los científicos empezaron a concienciarse del peligro que suponían estos compuestos en el desarrollo de procesos patológicos en animales y su posible conexión con las patologías humanas.

Asimismo, las micotoxinas son objeto de interés mundial debido a los efectos negativos que producen en la salud humana y animal, así como por las importantes pérdidas económicas que originan en la industria agroalimentaria. Estos compuestos son responsables de determinados síndromes en humanos y en animales de granja, denominados como "*micotoxicoxis"*. Entre sus efectos adversos para la salud destacan su incidencia en determinados tipos de cáncer y desórdenes neurológicos. Existe un gran número de micotoxinas; sin embargo, sólo unas pocas presentan efectos importantes en la seguridad alimentaria. La toxicidad puede ser muy variada dependiendo del tipo de toxina, de su concentración y del tiempo de exposición, pudiendo afectar al sistema nervioso central, a

los sistemas cardiovascular, pulmonar y al tracto digestivo. Algunas micotoxinas también pueden actuar como agentes cancerígenos, mutagénicos, teratógenos e inmunodepresores[34,35].

La contaminación de cereales con hongos productores de toxinas supone un gran riesgo sobre la población mundial, debido a la elevada contribución de los cereales como alimento básico de la dieta. Por ello, las micotoxinas constituyen uno de los riesgos alimentarios más importantes. La FAO (Organización de las Naciones Unidas para la Agricultura y la Alimentación) ha estimado que aproximadamente el 25% de los granos de cereales están contaminados con hongos productores de micotoxinas, lo que supone grandes pérdidas económicas[36].

Asimismo, la exposición a micotoxinas es más probable que ocurra en partes del mundo donde tienen pocos recursos sobre métodos de manejo y almacenamiento de alimentos y donde existe poca regulación para evitar la exposición a la población. Sin embargo, incluso en países desarrollados, donde existen unas buenas prácticas agrícolas y de manufactura, subgrupos específicos pueden ser vulnerables a la exposición de micotoxinas. Estos metabolitos pueden entrar en la cadena alimentaria directamente a través de productos vegetales como los cereales, el café, zumos de frutas y bebidas (vino y cerveza), o de forma indirecta a través de la dieta de los animales, mediante piensos (o pasto) contaminados con micotoxinas que pueden dejar residuos en la leche, la carne y otros productos.

Las micotoxinas han sido detectadas en productos alimentarios en muchas partes del mundo y han sido reconocidas como uno de los contaminantes más peligrosos en alimentos y piensos[36]. La producción de micotoxinas varía dependiendo de la zona geográfica. En efecto mientras que en los países europeos las aflatoxinas no son consideradas un gran problema, en Asía, África y ciertas partes de Australia, donde poseen un clima muy húmedo la existencia de aflatoxinas es muy común[37]. Aunque con el aumento del comercio internacional, no pasará mucho tiempo hasta que las principales micotoxinas se extiendan a todas las áreas del mundo[38].

Por otra parte, las micotoxinas no sólo son difíciles de definir, sino también lo son de clasificar. Esto es debido a que constituyen un grupo muy amplio de compuestos presentando diversas estructuras químicas y efectos biológicos y, a su vez, son producidas por un amplio número de especies fúngicas[39].

Se han descubierto alrededor de 400 micotoxinas. Estos compuestos, han sido clasificados de acuerdo a diversos criterios tales como la patología producida en hepatotóxicos, neurotóxicos, nefrotóxicos e inmunotoxinas. A su vez, también han sido clasificados en diferentes grupos genéricos tales como teratogénicos, mutagénicos, carcinógenos y alérgenos. Por otra parte, también se pueden clasificar en ciclopéptidos, terpenos, policetoácidos y metabolitos nitrogenados en función de su estructura química o de su origen biosintético. Aunque la clasificación más habitual hace referencia a sus similitudes estructurales y a su toxicidad[39].

La mayoría de las micotoxinas descritas están producidas por especies de los géneros *Aspergillus, Fusarium* y *Penicillium.* A continuación se va a hacer una descripción de las micotoxinas más importantes desde el punto de vista agroalimentario. Dentro de esta clasificación se encuentran: las aflatoxinas (B_1, B_2, G_1, G_2), las ocratoxinas, los tricotecenos (desoxinivalenol o vomitoxina, toxina T2), la zearalenona (ZEA), las fumonisinas y la patulina.

Las **aflatoxinas** son micotoxinas producidas por diferentes especies del género *Aspergillus (A. flavus, A. parasiticus y A. nominus)* y son, sin lugar a dudas, las más importantes y estudiadas debido a su elevada toxicidad. Se caracterizan por ser hepatotóxicas, carcinogénicas, teratogénicas y mutagénicas, afectando tanto a personas como a animales. Son un grupo de derivados difuranocumarínicos relacionados estructuralmente habiéndose identificado 18 tipos de aflatoxinas, de las cuales las más importantes y frecuentes son la $B_1(AFB_1)$, $B_2(AFB_2)$, $G_1(AFG_1)$ y $G_2(AFG_2)$. Estas han sido clasificadas como azules (B, del inglés *blue*) o verdes (G, del inglés *green*) en función de la fluorescencia que emiten bajo la luz ultravioleta.

La AFB_1 cuya estructura se muestra en la **Figura 11** es la más tóxica y ha sido clasificada como cancerígeno tipo 1 por la agencia internacional para la investigación contra el cáncer (IARC, *International Agency for Research on Cancer*). También es la más estudiada, existiendo gran cantidad de artículos científicos publicados relativos a esta micotoxina. De hecho, el término aflatoxinas hace referencia normalmente a la aflatoxina B1.

La contaminación por aflatoxinas es mayor en regiones tropicales donde las condiciones de humedad y temperatura favorecen su aparición, aunque también aparecen en zonas más templadas. Esta contaminación puede ocurrir en cualquier fase de la producción, antes de la cosecha o durante el almacenamiento. Aunque las aflatoxinas pueden encontrarse en muchos productos agrícolas, los mayores niveles de contaminación se han encontrado en semillas de algodón y maíz, cacahuetes, nueces, avellanas y otros frutos secos[40]. Debido al gran número de estudios existentes y, en base a su toxicidad y abundancia, están actualmente estrictamente reguladas.

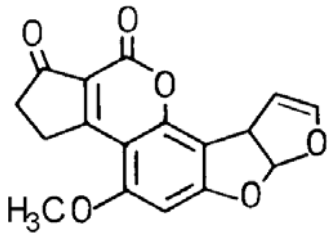

Figura 11. Estructura química de la aflatoxina B_1.

Las **ocratoxinas** son un grupo de micotoxinas producidas por especies *Aspergillus* y *Penicillium.* Fueron las primeras micotoxinas caracterizadas después de las aflatoxinas y están recibiendo una atención especial debido a su carácter nefrotóxico. El primer compuesto que se descubrió fue la ocratoxina A (OTA) en 1965, aislada de una cepa de *Aspergillus Ochraceus* durante un estudio para identificar nuevas micotoxinas. La ocratoxina A cuya estructura se muestra en la **Figura 12**, es actualmente la más estudiada y tóxica, por lo que está regulada en sus contenidos máximos en determinados alimentos. OTA puede encontrarse como contaminante común en cereales como cebada, avena, centeno, trigo, en subproductos de los mismos como harina, en legumbres, granos de café, y en productos

fermentados como vino y cerveza. OTA se ha relacionado con enfermedades graves en animales (nefropatía espontánea porcina, nefropatía aviar) y humanos (tumores en el trato urinario). Además las investigaciones realizadas permiten suponer que se trata de un agente cancerígeno para el hombre[41].

Existen otros tipos de ocratoxinas, que son derivados de OTA tales como ocratoxina B (OTB) y C (OTC), que son menos tóxicos. Asimismo, se encuentran las ocratoxinas α y β, que son productos de la hidrólisis de OTA y OTB.

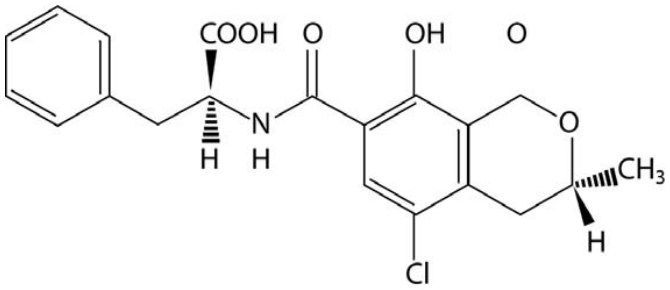

Figura 12. Estructura química de la ocratoxina A

Los **tricotecenos** son un grupo de micotoxinas relacionadas estructuralmente (sesquiterpenoides cíclicos), producidas por diversas especies del género *Fusarium.* Contaminan principalmente cereales de grano pequeño, como el trigo, la cebada y el maíz. Su ingestión produce un gran impacto en la salud de animales y humanos debido a que causan importantes efectos, incluyendo la inflamación de la piel, desórdenes digestivos, taquicardia, hemorragias severas en órganos internos y desórdenes nerviosos[42].

Los tricotecenos están clasificados en cuatro grupos (A, B, C y D) dependiendo de su estructura química. Dentro de los tricotecenos, destacan la toxina T-2 (grupo A), por ser altamente tóxica y el desoxinivalenol (DON) (grupo B), mucho más común aunque menos tóxico. El DON conocido comúnmente como vomitoxina, es el tricoteceno más frecuente. Es una micotoxina producida por *Fusarium graminearum*. Su estructura se muestra en la **Figura 13.** En Europa es uno de los contaminantes de cereales más comunes[43] y su ingestión produce una disminución en el consumo de alimentos (anorexia).

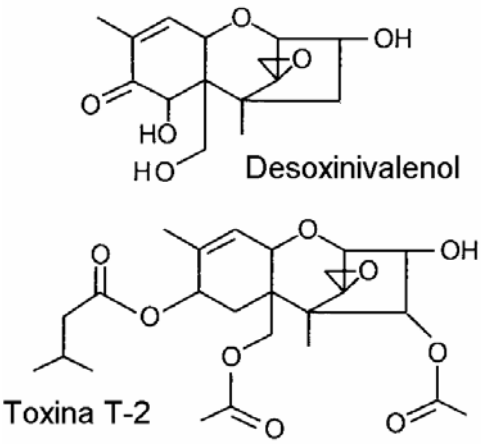

Figura 13. Estructuras químicas de DON y toxina T-2.

Las **fumonisinas** son micotoxinas producidas por varias especies del género *Fusarium (F. moniliforme, F. proliferatum y F. nygamai)*. Estas micotoxinas fueron descubiertas en 1988, y son conocidas por tener propiedades inductoras de cáncer. Pueden encontrarse como contaminantes comunes de productos agrícolas como maíz, higos secos, hierbas de té, leche, plantas medicinales y otros.

Las fumonisinas más comunes son la B1, B2 y B3. En particular la fumonisina B1, tiene gran importancia debido a ser la responsable de enfermedades graves en animales, como la leucoencefalomalacia (LEM) equina[44] o el edema pulmonar en cerdos[45]. Asimismo, varios estudios han demostrado la incidencia de las fumonisinas en el cáncer de esófago en humanos. Por todo ello, ha sido clasificada como posible cancerígeno (tipo 2B) por la agencia internacional para la investigación contra el cáncer (IARC, *International Agency for Research on Cancer*).

La Unión Europea ha establecido los contenidos máximos para estas micotoxinas en maíz y en productos derivados[46].

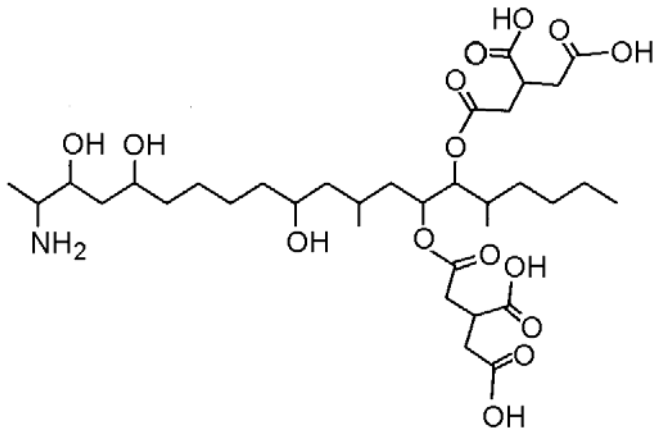

Figura 14. Estructura química de la fumonisina B_1.

Por otra parte, la **patulina** es una micotoxina producida por varias especies de *Penicillium* y *Aspergillus.* Esta micotoxina forma el grupo más pequeño de metabolitos tóxicos denominados policétidos. La patulina aparece en frutas dañadas superficialmente, ya que estas son vulnerables a la infección por hongos. Está considerada como la micotoxina más peligrosa en frutas, apareciendo principalmente en manzana y pera así como en productos derivados de las mismas, especialmente en zumos y en sidra.

Su ingestión afecta a las funciones del tejido gastrointestinal, hígado, riñón y al sistema inmunológico en general. Esta micotoxina está asociada con efectos genotóxicos, cancerígenos, también puede inducir la respuesta de estrés oxidativo de las células de mamíferos, generar especies reactivas de oxígeno e inducir apoptosis en células de leucemia humanas[38].

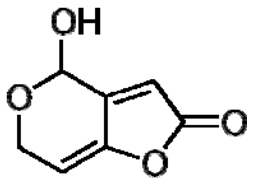

Figura 15. Estructura química de la patulina.

Finalmente, con respecto a la **zearalenona** decir, que esta micotoxina de gran importancia en el sector agroalimentario, ha sido elegida como analito para el desarrollo de esta Tesis y por tanto, se tratará de forma más exhaustiva en un epígrafe posterior.

Por otra parte, indicar que hasta el descubrimiento de las aflatoxinas, no se había dado a las micotoxinas la importancia necesaria. A partir de ese momento y debido al riesgo que suponían en la salud mundial, muchos países empezaron a establecer unos límites máximos para proteger a los consumidores de los efectos nocivos de los alimentos contaminados por micotoxinas[47]. La **Tabla 1** recoge de forma resumida estos aspectos.

Tabla 1. Trastornos producidos por las principales micotoxinas.

Micotoxinas	Trastornos
Aflatoxinas	Daño hepático, cirrosis, inducción de tumores
Desoxinivalenol (DON)	Rechazo del alimento, vómitos; inmunosupresión en cerdos y otros animales
Fumonisinas	Leucoencefalomalacia equina, edema pulmonar en cerdos, cáncer hepático en ratas
Ocratoxina A (OTA)	Nefropatía en cerdos y aves, acumulación en riñon, hígado y músculos
Patulina	Trastornos gastrointestinales y neurológicos, inducción de tumores
Zearalenona	Sindrome estrogénico en cerdos

Las primeras regulaciones fueron establecidas en países industrializados, siendo una especie de guías de asesoramiento más que de regulación. Con los años, el número de países que han ido estableciendo unos límites específicos de regulación de micotoxinas ha ido aumentando de 33 en 1981[48] a 56 en 1987[49], 77 en 1995[50] y 100 en 2003[51]. A partir de 2004, en la Unión Europea empezó a extenderse la necesidad de establecer unas regulaciones armonizadas en materia de micotoxinas en alimentos y piensos. Actualmente, instituciones nacionales e internacionales y organizaciones de todo el mundo (FDA, WHO y la FAO) han reconocido el gran riesgo que supone sobre la salud de animales y humanos la ingestión de alimentos contaminados con micotoxinas, y han abordado el problema adoptando límites de regulación sobre las principales micotoxinas. A su vez, la necesidad de establecer una regulación ha incrementado el interés en el desarrollo de métodos analíticos para identificar y cuantificar micotoxinas en alimentos. Además, hay que tener en cuenta que las micotoxinas son a menudo producidas en concentraciones de trazas, por lo que se hace preciso el empleo de métodos de alta sensibilidad.

I.3 ANTECEDENTES BIBLIOGRÁFICOS SOBRE LA DETERMINACIÓN DE ZEARALENONA.

La estructura química de la zearalenona (ZEA), 6-(10-hidroxi-6-oxo-trans-undecenil)-ácido β-resorcíclico-lactona, se muestra en la **Figura 16**. También conocida como toxina F-2, es una micotoxina no esteroidea, con actividad estrogénica y es producida por varias especies del género *Fusarium*. Asimismo, se distribuye por todo el mundo, variando su incidencia y niveles de contaminación en función de los factores ambientales en los que se encuentra el hongo y puede contaminar a la mayoría de los cereales de grano pequeño tales como cebada, maíz, avena y trigo, y en consecuencia, aparecer en productos derivados de los mismos.

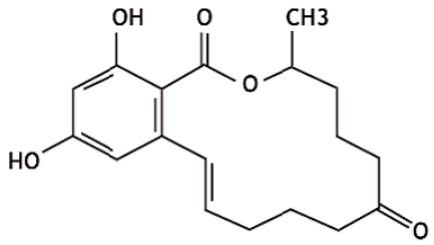

Figura 16. Estructura química de la zearalenona.

La ZEA es una micotoxina que está adquiriendo una gran importancia hoy en día debido a su presencia en un gran número de alimentos y al riesgo que supone en la salud animal y humana. Su toxicidad se debe a su actividad estrogénica, originada por su similitud con la estructura química de algunos estrógenos. Tanto la ZEA como sus derivados pueden dar lugar a efectos estrogénicos tales como infertilidad, hipertrofia mamaria, vulvovaginitis (fundamentalmente en cerdos) debido a que reaccionan con los receptores estrogénicos[52]. Únicamente a elevadas concentraciones y a muy pequeña escala, pueden producir efectos inmunosupresores en humanos[53] o hiperplasia endometrial y adenocarcinomas[54]. Dada su toxicidad, ha surgido la necesidad de establecer límites de contenido máximo en los alimentos y mejorar los procedimientos de control de esta micotoxina.

Según fuentes de la FAO, en 2004, la ZEA estaba regulada por 6 países en 1996, pero pasó a estar regulada por 16 países en 2003 en alimentos y piensos. Sin embargo no existe un límite máximo internacional armonizado para dicha micotoxina. Los niveles máximos

vigentes establecidos por la Comisión Europea son 20, 75 y 100 µg Kg^{-1} en alimentos infantiles, harinas de cereales y en cereales no elaborados, respectivamente[52,56,57]. Debido a las importantes implicaciones de ZEA en la salud humana y animal mencionadas anteriormente, así como los aspectos económicos que origina, ha surgido una extensa investigación hacia el desarrollo de métodos sensibles y específicos para la rápida detección de ZEA en muestras de alimentos y piensos.

La existencia de regulaciones normativas junto a las pérdidas económicas que supone la presencia de micotoxinas en alimentos, y su impacto en la salud humana y animal, han hecho necesario el desarrollo de distintas estrategias metodológicas para determinar sus niveles de contaminación y evitar su consumo. La AOAC (*Association of Official Analytical Chemists*) describe hasta 45 métodos para la determinación de micotoxinas[58]. La metodología empleada incluye un exhaustivo paso de extracción, una etapa de limpieza para reducir o eliminar los componentes no deseados de la matriz, una etapa de separación y la detección. Habitualmente, esto se lleva a cabo mediante una columna de inmunoafinidad para la etapa de limpieza, seguida de las técnicas de separación cromatográficas en combinación con diferentes sistemas de detección. Actualmente y como métodos rápidos de cribado, además de la cromatografía en capa fina, los métodos inmunoanalíticos (ELISA) están siendo ampliamente utilizados gracias a que hacen innecesaria la etapa de limpieza y preconcentración de los analitos[59-64].

A continuación, se comentarán los antecedentes bibliográficos relacionados con la determinación de ZEA en dos partes claramente diferenciadas; en primer lugar, aquellos relacionados con métodos no inmunoanalíticos y, seguidamente, los relacionados con los métodos inmunoanalíticos.

Por otra parte y en referencia directa a los antecedentes bibliográficos relacionados con los métodos inmunoanalíticos, aquellos correspondientes a estrategias ELISAs en formato clásico y a estrategias basadas en el desarrollo de inmunosensores se encuentran de forma detallada en las introducciones de los artículos científicos publicados y recogidos en los capítulos III.1 y III.2 páginas 65 y 73, respectivamente. Por su parte, debido a las particularidades y novedad que presentan, los antecedentes bibliográficos sobre

inmunoanálisis electroquímico empleando sistemas microfluídicos para la detección de micotoxinas en general, se ha creído conveniente estudiarlos de forma detallada en el capítulo III.3 página 83 y que ha constituido un trabajo de revisión bibliográfica para tal fin.

Sin embargo, en aras de obtener una visión de conjunto y general, permitiendo una mejor contextualización científica del trabajo desarrollado, a continuación se muestran ejemplos relevantes del conjunto bibliográfico consultado en referencia directa a la determinación de la ZEA.

I.3.1 Determinación de zearalenona mediante métodos no inmunoanalíticos

Al igual que ocurre en el análisis de otras micotoxinas, son las técnicas cromatográficas tales como TLC[65,66], GC[67] y HPLC[68-85] las que se han aplicado con mayor profusión para el análisis de zearalenona. Sin embargo, estos métodos requieren equipos caros y son muy laboriosos, ya que necesitan realizar complicados procedimientos de limpieza, etapas de preconcentración, y en algunos casos es necesario derivatizar la muestra. A continuación, de forma resumida, se comentarán los aspectos analíticos más relevantes de los trabajos encontrados.

Schaafsma llevó a cabo el análisis en muestras conteniendo ZEA mediante TLC utilizando placas de sílice como adsorbente y una combinación de disolventes tales como metanol, n-hexano, tolueno y acetato de etilo. Tras la excitación con radiación UV de 274 nm se observa la fluorescencia de ZEA. El LOD para alimentos con esta técnica se sitúa en torno a 200 ppb ($\mu g\ Kg^{-1}$)[66].

La cromatografía de gases acoplada a la espectrometría de masas (GC-MS) ha resultado ser un método muy útil para la determinación de esta y otras micotoxinas de forma simultánea. El límite de detección de ZEA en muestras fortificadas de cereales fue 5 ppb[67]. Por otra parte, la introducción de la ionización química a presión atmosférica (APCI) por parte de Rosenberg acoplada a un sistema de cromatografía líquida y espectrometría de masas (LC-APCI-MS) permitió la determinación de varias muestras alimentarias (contaminadas y fortificadas) a una concentración de 0.12 ppb[68].

Por su parte, las columnas de inmunoafinidad (IACs) han mejorado las etapas de extracción y purificación de la muestra. La utilización de estas columnas para purificación y preconcentración seguida de la separación por HPLC con detección fluorimétrica, ha sido aplicada para el análisis de zearalenona y **α**-zearalenol en cereales y piensos para animales[69]. Las recuperaciones obtenidas para muestras fortificadas con ZEA y **α**-ZOL comprendían un intervalo de 89 a 110% con coeficientes de variación entre 5 y 11 %. Del mismo modo, Howell y Taylor (1981)[70] llevaron a cabo el análisis de aflatoxinas, ocratoxina A y zearalenona en piensos. El LOD obtenido fue de 1 ppb para estas micotoxinas.

El acoplamiento HPLC con MS en tándem resulta una poderosa herramienta para la caracterización e identificación de micotoxinas. El análisis de ZEA mediante LC-MS/MS en el orden de µg/kg ha sido recientemente presentado en los trabajos de Berthiller y Cavaliere. En el primero de los estudios, la determinación se realizó mediante LC-APIC-MS/MS, obteniéndose un LOD de 0.9 ppb[71]. En el trabajo de Cavaliere se determinaron ocho tricotecenos, tres fumonisinas, zearalenona y **α**-zearalenol en harina de trigo mediante LC-ESI-MS/MS. Las condiciones de LC y MS se variaron hasta encontrar el mejor compromiso en términos de sensibilidad y separación, que se obtuvo utilizando una columna C_{18} (45º C) y un gradiente de fase móvil de metanol/agua con 10 mmoL de tampón (pH 3.8). Los límites de detección del método estaban comprendidos en el intervalo 2-14 ppb, siendo de 10 ppb para el caso concreto de la zearalenona[72].

Otra técnica de separación empleada en la detección de ZEA ha sido la electroforesis capilar empleando ciclodextrinas que permiten aumentar la fluorescencia natural de ZEA. Mediante este método se han obtenido límites de cuantificación de 5 ppb en maíz y recuperaciones cuantitativas (103.1 ± 8.5 %, n =20)[86].

I.3.2 Determinación de zearalenona mediante métodos inmunoanáliticos no electroquímicos

Tal y como se ha comentado previamente, en los últimos años, los métodos inmunoanalíticos, han llegado a ser una alternativa analítica importantísima para la monitorización de micotoxinas en general y de ZEA en particular. Sus excelentes

características en cuanto a rapidez, facilidad de operación, sensibilidad, minimización de la etapa de limpieza y bajo coste han permitido su gran desarrollo en esta área. Sin embargo, es preciso señalar que, en algunos casos, pueden verse influenciados por el efecto matriz y una posible sobreestimación[65].

La ZEA ha sido analizada por diferentes métodos inmunoanalíticos, incluyendo ELISAs[87-92], inmunoensayo de polarización fluorescente[93], inmunoensayo en tiras[94] e inmunosensores de flujo continuo[95]. Algunas técnicas más recientes incluyen un inmunoensayo sándwich abierto, en el cual el antígeno induce una mayor interacción V_H/V_L[96], inmunoensayos de flujo lateral basados en oro coloidal[97], métodos ELISA que hacen uso de nuevos anticuerpos scFV (*single chain fragment variable*)[98] y también de nuevos anticuerpos monoclonales específicos[99]. Adicionalmente, se han desarrollado kits comerciales basados en métodos ELISA para la determinación de ZEA en alimentos y piensos. Por otra parte, también se han desarrollado polímeros de impresión molecular (MIPs, *del inglés molecular imprinted polymers*) específicos para ZEA[100-101], los cuales evitan los problemas de estabilidad asociados al uso de disolventes orgánicos, muy comunes en inmunoensayo. Sin embargo, estos polímeros proporcionan una sensibilidad más baja que los métodos inmunoanalíticos.

El grupo de investigación de Petska ha descrito dos métodos ELISA para la determinación de ZEA en cereales con una única extracción con metanol-tampón fosfato salino-dimetilformamida[87,88]. Los valores de recuperación obtenidos para varias concentraciones dieron un valor medio del 97%, siendo comparables a los obtenidos mediante HPLC. En ambos casos, el límite de detección fue de 1 ppb.

Barna-Vetró et al., han publicado un método ELISA con anticuerpos monoclonales específicos para ZEA y que recurre a un 89% de acetonitrilo para la extracción y diluciones (1:25 o 1:50) para disminuir el efecto de matriz[89]. En estas condiciones se obtuvo una recuperación del 91% para muestras de diferentes cereales fortificadas desde 50 a 500 ppb, así como uno LOD para ZEA en el tampón de 0,2 ppb.

Del mismo modo, el grupo de Tanaka han descrito un ELISA competitivo, para la determinación de zearalenona en muestras fortificadas de cebada y Job's Tears[90]. Se

emplearon dos métodos de extracción con metanol-agua-diclorometano y metanol-agua, que no presentaron diferencias significativas con el método de HPLC empleado para su comparación. El LOD para ZEA fue 10 ppb.

Otros trabajos han llevado a cabo el análisis de un conjunto elevado de muestras (hasta 89) de maíz mediante un método ELISA con detección espectrofotométrica, demostrándose la viabilidad del mismo mediante comparación con un método de HPLC combinado con columnas de inmunoafinidad[91]. Los resultados analíticos arrojaron una buena correlación entre ambos métodos (R^2= 0.9258), buenos niveles de recuperación (102%) y buenos LODs (3 ppb).

Finalmente y en referencia directa a inmunoanálisis no electroquímicos, Urraca y colaboradores han desarrollado un sistema de flujo basado en un ELISA competitivo y detección fluorescente para la determinación de ZEA en muestras fortificadas de trigo y pienso de cerdos[95]. El proceso de extracción se llevó a cabo mediante la técnica de extracción acelerada con disolventes. La validación del método se realizó utilizando un material de referencia certificado de maíz y por comparación con HPLC con detección fluorescente. El límite de detección obtenido fue de 0.007 ppb, significativamente inferior a los presentados anteriormente.

I.3.3 Determinación de zearalenona mediante métodos inmunoanáliticos electroquímicos

En primer lugar, es preciso señalar que el moderado carácter electroactivo de la ZEA ha permitido llevar a cabo su detección electroquímica directa (Ramírez et al[102]., 2005; Zougahg et al[103]., 2008), aunque la sensibilidad alcanzada no fue muy elevada. En efecto, Ramirez et al., llevaron a cabo la determinación de ZEA en muestras fortificadas, mediante preconcentración en la superficie de un electrodo de carbono vitrificado y su posterior detección por voltamperometría de onda cuadrada (SWV). En este caso, el límite de detección obtenido fue de 30 ppb[102]. Por otra parte, Zougagh et al., han desarrollado un método de *screening* rápido y sencillo para determinar ZEA y derivados en muestras de harina de maíz[103]. Este método comprende una extracción mediante fluidos supercríticos,

seguido de preconcentración por cromatografía, con detección electroquímica mediante flujo continuo. El LOD obtenido fue de 16 ppb.

A su vez, también se han desarrollado diferentes inmunosensores electroquímicos para la detección de micotoxinas en diferentes tipos de muestras. La principal desventaja de los inmunosensores basados en electrodos convencionales es la dificultad de regeneración de la fase de inmunoreconocimiento. Con el fin de superar este problema y conseguir dispositivos desechables y más sensibles, se han descrito inmunosensores basados en electrodos serigrafiados. Las ventajas de estos inmunosensores electroquímicos son, por una parte su miniaturización y portabilidad, y por otra, su bajo coste el cual permite su producción en masa. Por ello, se pueden encontrar inmunosensores para algunas de las principales micotoxinas (aflatoxinas[104-107], ocractoxina[108-109], DON[110]). Sin embargo existen muy pocos ejemplos de inmunosensores electroquímicos para la determinación de ZEA.

A su vez, la modificación de electrodos con nanotubos de carbono (CNTs) mejora notablemente la sensibilidad de los inmunosensores, debido al aumento de la relación superficie/volumen y a una mayor cinética en la transferencia de electrones para una amplia gama de sustancias electroactivas. Recientemente, Panini et al., han publicado un inmunosensor acoplado a un electrodo de carbono vitrificado (GCE) modificado con MWCNTs e integrado en un sistema de flujo continuo para la determinación rápida y sensible de ZEA en muestras de maíz[111]. Para ello, los anticuerpos anti-ZEA fueron inmovilizados en un disco rotatorio produciéndose la competición entre ZEA y ZEA-HRP por su unión con los anticuerpos. El tiempo total del ensayo fue de 15 minutos y el LOD obtenido de 0.77 ppb.

Recientemente, la combinación de la tecnología microfluídica y los inmunoensayos ha demostrado ser una poderosa alternativa en el análisis de alimentos, ya que esta combinación aúna de forma sinérgica, por una parte, el gran poder analítico de los sistemas microfluídicos (disminución de los tiempos de análisis, reducción del consumo de reactivos y muestras, posibilidad de llevar a cabo análisis paralelos y multiplexados y su alta compatibilidad con la automatización); y por otra, la elevada sensibilidad y especificidad asociada a la unión antígeno-anticuerpo. En base a esto, Panini y colaboradores han

desarrollado un inmunosensor microfluídico acoplado a un electrodo de oro para la cuantificación de ZEA en piensos de animales[112]. La detección de ZEA se lleva a cabo mediante un inmunoensayo competitivo directo que hace uso de anticuerpos anti-ZEA inmovilizados sobre partículas magnéticas. La ZEA presente en la muestra compite con su conjugado enzimático (ZEA-HRP) por los sitios de unión con los anticuerpos inmovilizados. La peróxidasa (HRP) en presencia del H_2O_2 cataliza la oxidación del 4-TBC, cuya reducción electroquímica en la superficie del electrodo de oro tiene lugar a 0V. El LOD obtenido fue 2.56 ppb.

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II HIPÓTESIS DE PARTIDA, OBJETIVO Y PLAN DE TRABAJO

En esta sección se describe la problemática que se abordará en este trabajo de Tesis.

Asimismo, se plantea el objetivo general y las estrategias llevadas a cabo para conseguir dicho objetivo.

Conscientes y sensibles de que uno de los campos más importantes en seguridad alimentaria, debido a los efectos adversos que originan en la salud de personas y animales, es la detección y el control de micotoxinas a niveles extremadamente bajos; este trabajo de investigación se gesta y nace con la vocación firme de aportar nuevas soluciones a tal problemática. Con tal motivo, se han diseñado nuevas alternativas con prestaciones analíticas mejoradas a las ya existentes haciendo uso de una de las herramientas analíticas más poderosas que existe, el inmunonanálisis (alta selectividad, especificidad, sensibilidad), en combinación con una detección de altas prestaciones analíticas, la detección electroquímica (alta sensibilidad, inherente miniaturización, bajo coste). Todo ello ha dado en denominarse Inmunoanálisis Electroquímico.

La **Hipótesis de partida** de este trabajo de investigación tiene su razón de ser en los siguientes aspectos analíticos. En primer lugar, en la baja selectividad y sensibilidad que inherentemente ofrece la detección electroquímica directa de la micotoxina ZEA, donde la literatura más relevante ofrece límites de detección muy superiores a los requeridos por la legislación. En segundo lugar, en la inherente selectividad y sensibilidad que ofrecen los métodos inmunoanalíticos, comparables a los obtenidos mediante técnicas hibridadas de cromatografía y espectrometría de masas. En último lugar, y en comparación con dichas técnicas hibridadas, los métodos inmunoanalíticos electroquímicos resultan ser más sencillos, económicos, fáciles de miniaturizar y en consecuencia portátiles, ofreciendo un bajo consumo de muestra y reactivos, todo ello sin detrimento alguno de sus prestaciones analíticas dado que generan una información analítica plena acorde a los requerimientos exigidos por la legislación vigente. Este hecho confiere a los métodos inmunoanalíticos un alto valor añadido como herramientas de cribado con alto protagonismo en el análisis descentralizado y, sin lugar a dudas, como extraordinario complemento a las técnicas de confirmación basadas en las ya mencionadas cromatografías hibridadas a espectrometría de masas.

Por todo ello y como consecuencia natural, el **objetivo central** de esta Tesis Doctoral ha sido diseñar, desarrollar y evaluar nuevas estrategias analíticas miniaturizadas basadas en inmunoanálisis electroquímico soportado sobre partículas magnéticas, de prestaciones

analíticas mejoradas que den respuesta rápida, fiable y sencilla a la problemática regulada por la legislación vigente como alternativa fehaciente a aquellas ya existentes que emplean instrumentación analítica cara y sofisticada.

En este sentido, y en un mayor grado de concreción, los objetivos parciales, todos ellos para llevar a cabo la detección y el control de ZEA en muestras seleccionadas como representativas han sido:

1-Optimización, desarrollo y evaluación analítica de un ELISA electroquímico convencional en placa como nueva herramienta analítica que permita asegurar la detección y el control de la micotoxina zearalenona.

2-Diseño, desarrollo y evaluación analítica de un inmunosensor electroquímico miniaturizado soportado sobre partículas magnéticas empleando electrodos serigrafiados desechables.

3-Diseño y propuesta de una nueva estrategia para integrar todas las etapas del inmunoanálisis electroquímico objeto de estudio en un sistema microfluídico de diseño sencillo como plataforma sensora de nueva generación.

4-Propuesta de nuevas estrategias de simplificación metodológica para llevar a cabo la calibración y el análisis de las muestras de forma fiable, rápida, sencilla y descentralizada.

5-Búsqueda de sinergias entre las estrategias de miniaturización y simplificación propuestas.

6-Discusión comparada de las estrategias desarrolladas y propuesta de la aproximación analítica más viable.

Para alcanzar los objetivos anteriormente expuestos, y empleando siempre un material certificado de referencia y muestras de alimentos seleccionadas como representativas para el estudio de evaluación analítica de cada una de las estrategias propuestas, se desarrolló el siguiente plan de trabajo acorde, fundamentalmente a dos grandes etapas metodológicas:

En la **primera etapa metodológica**, se llevó a cabo el diseño y optimización de un método inmunoanalítico soportado sobre partículas magnéticas con detección electroquímica en

electrodos serigrafiados de carbono para la detección y el control de ZEA en muestras alimentarias infantiles. La reacción inmunológica se basa en un ensayo competitivo en el cual las partículas magnéticas son utilizadas como soporte de inmovilización y la enzima peroxidasa (HRP) se utiliza como marcador enzimático.

En la **segunda etapa metodológica** y en aras de explotar los beneficios inherentes de la miniaturización (bajo consumo de reactivos y muestra, portabilidad, simplicidad y posibilidades de descentralizar los análisis), se propusieron dos estrategias miniaturizadas de diferente naturaleza y abordaje: por una parte, el diseño y desarrollo de un inmunosensor sobre transductores de carbono serigrafiados y, por otra, el diseño y desarrollo de una plataforma microfluídica integrando todas las etapas requeridas para llevar a cabo el inmunoanálisis electroquímico objeto de estudio.

Con tal motivo, en este sentido se llevó a cabo el desarrollo de un inmunosensor electroquímico soportado en partículas magnéticas empleando electrodos de carbono serigrafiados desechables proponiendo por primera vez un protocolo de calibración simplificada que evitara la construcción de la curva de calibración clásica.

Con respecto a la estrategia final cuya vocación fue la integración de todas las etapas requeridas para llevar a cabo el inmunoanálisis electroquímico objeto de estudio en una plataforma microfluídica, ésta se llevó a cabo en dos partes perfectamente diferenciadas.

En la primera de ellas, se evaluó la capacidad analítica de la plataforma microfluídica para monitorizar electroquímicamente el producto obtenido en el ELISA en placa incorporando un protocolo de calibración integrada, empleando adecuadamente los dos reservorios de la plataforma microfluídica.

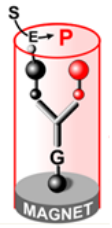
Como consecuencia natural y acorde al objetivo central de este trabajo, finalmente y en esta etapa metodológica, se llevó a cabo el estudio de las posibilidades de integración de las etapas inmunoanalíticas en un sistema microfluídico: la etapa de bio-reconocimiento en formato competitivo y la correspondiente a la incubación enzimática y posterior monitorización electroquímica. La aplicación estratégica de campos eléctricos en los distintos reservorios, permitió llevar a cabo las etapas inmunoquímicas en diferentes zonas

del sistema microfluídico mediante la adecuada manipulación de fluidos y el empleo de partículas magnéticas. Asimismo, se propuso un protocolo de calibración simplificada que permitió la evaluación de las prestaciones analíticas de esta plataforma sensora de nueva generación.

III RESULTADOS Y DISCUSIÓN

En este capítulo se presentan detallada y cronológicamente todos los resultados obtenidos en el desarrollo de esta Tesis doctoral.

III.1 ARTÍCULO 1:



Desarrollo de un ELISA electroquímico en placa para la detección y control de ZEA en alimentos infantiles

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Electrochemical immunoassay using magnetic beads for the determination of zearalenone in baby food: An anticipated analytical tool for food safety

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ABSTRACT

In this work, electrochemical immunoassay involving magnetic beads to determine zearalenone in selected food samples has been developed. The immunoassay scheme has been based on a direct competitive immunoassay method in which antibody-coated magnetic beads were employed as the immobilisation support and horseradish peroxidase (HRP) was used as enzymatic label. Amperometric detection has been achieved through the addition of hydrogen peroxide substrate and hydroquinone as mediator.

Analytical performance of the electrochemical immunoassay has been evaluated by analysis of maize certified reference material (CRM) and selected baby food samples. A detection limit (LOD) of 0.011 $\mu g\,L^{-1}$ and EC_{50} 0.079 $\mu g\,L^{-1}$ were obtained allowing the assessment of the detection of zearalenone mycotoxin. In addition, an excellent accuracy with a high recovery yield ranging between 95 and 108% has been obtained. The analytical features have shown the proposed electrochemical immunoassay to be a very powerful and timely screening tool for the food safety scene.

1. Introduction

Mycotoxins are potent toxins produced by fungi as secondary metabolites. Zearalenone (Zea) is a nonsteroidal oestrogenic mycotoxin produced by several species of *Fusarium* [1,2]. Storage of *Fusarium*-infected cereals under high humidity conditions or at high temperatures produces elevated levels of Zea in different cereals such as maize, barley, oats, wheat, rice, and sorghum in temperate regions around the world [3]. In addition, Zea is a stable compound that has been found on grains in the field during harvest, in commercial grain processing, in the storage facilities of food or feedstuff containing the grain, and it does not degrade at high temperatures [1,4]. The acute toxicity of Zea is relatively low (oral LD_{50} >20,000 mg kg^{-1} body weight) upon oral administration in animals [5]. Nevertheless, this mycotoxin causes alterations in the reproductive tract of both laboratory and domestic animals and, in humans, it has been associated with precocious puberty, hyperplasic and neoplastic endometrium, and human cervical cancer [6,7]. The oestrogenic properties mainly associated with the toxic effects of Zea and related compounds have recently been reviewed [5].

Six countries established regulations for Zea in food and feed in 1996. In 2004, there were a total of 16 countries (FAO 2004) [8], which clearly indicates its significance. However, the maximum tolerable levels differ greatly between countries and a common international maximum limit has not been set for Zea in foodstuff. The current maximum levels set by the European Commission are 20, 75, and 100 $\mu g\,kg^{-1}$ for baby food, cereal flour, and unprocessed cereals, respectively [9–11]. Due to the important implications surrounding the effect of Zea on human and animal health that were mentioned earlier as well as economic aspects, extensive research has been conducted to develop sensitive and specific methods for rapid and economic Zea detection in food and feed samples.

Recently, mycotoxin analysis was extensively reviewed by Krska [12–14]. The main analytical techniques used for Zea analysis in cereals include thin-layer chromatography (TLC) [15,16]; gas chromatography–mass spectrometry (GC–MS) [17]; high-performance liquid chromatography (HPLC) using different detection principles such as UV diode array [18,19], mass spectrometry [20–26], fluorescence [27–34] as well as electrochemical detection [35]; and capillary electrophoresis [36]. These methods, however, require expensive equipment, complicated cleanup procedures, pre-concentration steps, and skilled operators.

Immunoassays, on the other hand, have proven to be an excellent analytical alternative as they are sensitive, specific, inexpensive, rapid, simple, and high-throughput methods that can be used as an "alarm" to detect different chemical contaminants in a wide variety of food matrices [37]. Immunoanalytical techniques that have been used for Zea detection include enzyme-linked immunosorbent assay (ELISA) methods [38–43], fluorescence polarisation immunoassay (FPIA) [44], dipstick immunoassay [45], and an automated flow-through immunosensor [46]. More recent techniques include an open sandwich immunoassay in which the

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antigen induced an enhanced V_H/V_L interaction [47], a colloidal gold-based lateral-flow immunoassay [48], ELISA methods which made use of a new scFv antibody [49], and a new specific monoclonal antibody [50] that were developed for detecting Zea in cereal samples. Additionally, a number of commercial ELISA-based kits have been developed for detecting Zea in food and feed products.

Alternatively, specific molecular imprinted polymers have also been developed with recognition properties for Zea which are not affected by storage limitations and stability problems usually associated with immunoassays when organic solvents are used. However, lower sensitivity is reached compared to immunoassay [51,52].

Electrochemical immunoassays are receiving special focus because of their inherent simplicity, suitability for mass production, low-cost fabrication, and high sensitivity, as well as their large number of labels, including enzymes and electroactive molecules, and their nanotechnology applications, such as for gold particles and semiconductor crystals. Although direct electroanalytical detection of Zea has also been studied [53,54], the sensitivity reached is not very high. Surprisingly, however, no electrochemical immunoassays for Zea have been reported to date even though this detection principle is one of the most attractive alternatives to optical detection.

In addition, magnetic beads have proven to be a powerful and versatile tool in the development and applications of immunoassays for food contaminants [55–58]. It has been reported that the use of immunobeads improves the performance of the immunological reaction due to an increase in the surface area, as well as the assay kinetics are achieved more rapidly since the beads are in suspension and the probability that antibody-coated magnetic beads meet the analyte is very high, keeping the solution under stirring. Furthermore, the magnetic beads can easily be manipulated through the use of permanent magnets, and the matrix effect is also minimised due to improved washing and separation steps despite this increased surface area. Therefore, this strategy allows the analysis of samples to be made without any pre-enrichment, purification, or pre-treatment steps as is normally required.

As consequence of all statements discussed above, this paper explores the combination of a magnetic particle based immunoassay with electrochemical detection for the first time to determine Zea in different food matrices. The electrochemical immunoassay was evaluated through the determination of Zea in a maize certified reference material (CRM) and in selected baby food containing cereals.

2. Experimental

2.1. Chemicals and immunochemicals

Zearalenone (Zea), bovine serum albumin (BSA), hydrogen peroxide (30%), hydroquinone (HQ), benzoquinone (BQN) and Tween 20 were purchased from Sigma–Aldrich (Madrid, Spain). Standard Zea solutions were prepared daily by diluting stock solutions (5 mg mL^{-1} in acetonitrile) in phosphate buffer saline (PBS). Anti-zearalenone monoclonal antibody and the enzyme tracer Zea conjugated to HRP were supplied by Soft Flow Biotechnology (Gödöllõ, Hungary). All of the other reagents were of the highest available grade.

Superparamagnetic polymer beads with protein-G covalently coupled to the surface (Dynabeads® protein-G) were supplied by Invitrogen Dynal AS (Oslo, Norway).

Unless otherwise indicated, PBS or PBS modified with Tween and BSA was used. The composition of the PBS solution was phosphate buffer 10 mmol L^{-1} pH 7.5 with 0.8% (w/v) NaCl. In the other PBS solution, 0.05% (v/v) Tween and 0.1% BSA (dilution buffer) or 1% BSA (blocking buffer) were also added. Citrate–phosphate buffer (24.5 mmol L^{-1} citric acid and 51.7 mmol L^{-1} dibasic sodium phosphate) pH 5.0, was used for washing magnetic beads and the antibody capture procedure according to the manufacturer's protocol. All buffer solutions were prepared with Milli-Q water.

2.2. Samples

Zea in maize certified reference material (83 ± 9 μg kg^{-1}) was purchased from Fluka (BCR®). Baby food cereal (Blevit multicereales) containing a mixture of wheat, rice, maize, barley, oats, sorghum, rye, and soya flour was purchased from a local pharmacy (Madrid, Spain). Cereal milkshakes (Puleva) containing a mixture of wheat, rice, maize, barley, oats, and rye were purchased from a local retail store (Madrid, Spain).

2.3. Equipment

Amperometric measurements were performed with an Eco-Chemie Autolab PGSTAT. A three-electrode setup comprised of a platinum auxiliary electrode, an Ag/AgCl (3 M KCl) reference electrode, and carbon thick-film screen-printed electrodes as a working electrode were used in a homemade 1.8 mL glass cell. Magnetic separation during the washing steps was performed using a magnetic separation rack (Ohmicron). An ultrasonic bath from Sonic Vibra-Cell was used in the extraction procedure. The competitive curves were analyzed with a four-parameter logistic equation using suitable software (Graph Pad Prism 5).

2.4. Immunoassay procedure

The immunoassay was developed on the basis of a competition scheme, in which the mycotoxin zearalenone and an enzyme-labelled derivative compete for the binding sites of the specific antibody. Protein-G covalently bound to magnetic particles acts as an oriented immobilisation support for the capture of the anti-mycotoxin antibody. After the target molecular recognition event takes place, the extent of the affinity reaction is evaluated by the electrochemical detection of the mediator directly related to the activity of the enzyme tracer (HRP–zearalenone). The scheme of this procedure is shown in Fig. 1.

The competitive immunoassay was performed on 2 mL glass tubes or ELISA microplate wells. After each incubation or washing step, the magnetic beads were separated from the supernatant by placing the glass tubes or microplate wells into a magnetic rack until the magnetic beads migrated to the side walls and the liquid, now clear, was then removed.

Initially, the Dynabeads® protein-G magnetic particles were washed with citrate–phosphate buffer pH 5.0 according to the manufacturer's protocol in order to eliminate the storage buffer and to condition them for the IgG capture procedure. In this way, a volume of 2 μL of Dynabeads® was introduced into the well and washed twice with citrate–phosphate buffer. After precipitation of the beads by placing the well into the magnetic rack, the supernatant was removed. Then, 50 μL of specific antibody (5 μg mL^{-1}) prepared in citrate–phosphate buffer pH 5.0 was added to the magnetic beads. The solution was stirred gently for 30 min at room temperature to obtain antibody-modified beads. The washing step was repeated five times with 200 μL of PBST which allowed any unbound antibody to be eliminated. The competitive assay was performed by re-suspending the anti-mycotoxin antibody-coated beads in 50 μL of a mixture of either zearalenone standard solution or sample and the enzyme tracer (final dilution 1:200 in PBST–BSA 0.1%). The competitive immunological reaction was allowed to proceed with gentle stirring for 30 min at room temperature. The magnetic beads were again washed five times with 200 μL of PBST

to eliminate any unbound species. Finally, Ag–Ab complex beads were re-suspended in 100 μL of solution containing the mediator (hydroquinone, HQ, 800 μM) and the enzymatic substrate (hydrogen peroxide, 600 μM). Hydrogen peroxide was used at this concentration to ensure that all enzymatic molecules were substrate bound and that Vmax (according to the Michaelis–Menten model) was reached. The enzymatic reaction was then allowed to proceed for 25 min, after which 100 μL of supernatant was removed and added to the electrochemical cell containing 1.8 mL PBS, pH 7.4. The response was determined at −0.250 V (previously optimised by cyclic voltammetry) carbon screen-printed working electrode where the reduction of the enzymatically oxidised mediator HQ took place.

2.5. Calibration protocol

The standard curve for Zea was fitted to a four-parameter logistic equation using the Graph Pad Prism 5 software according to the equation [56]

$$Y = A + \frac{B - A}{1 + 10^{[\log EC_{50} - x]\text{Hill slope}}}$$

where A is the Y value at the bottom plateau of the curve (minimum intensity current corresponded to infinite concentration), B is the Y value at the top plateau of the curve (maximal intensity current corresponded to zero concentration), EC_{50} is the antigen concentration necessary to halve the current signal, and Hill slope is the slope of the linear part of the curve.

2.6. Sample extraction procedure

Maize certificate reference material (Zea levels = 83 ± 9 μg kg^{-1}) was extracted under the same extraction method but using two different combinations of solvents. In procedure A, adapted from literature [46], 1 g of sample was extracted with 4 mL of acetonitrile:methanol (50:50, v/v) for 90 min at room temperature in an ultrasonic bath. In procedure B, taken from CRM protocol, 1 g of sample was extracted with 4 mL of acetonitrile:water (75:25, v/v) for 90 min at room temperature in an ultrasonic bath. In both cases the resulting mixture was centrifuged at 4000 rpm for 10 min and the supernatant (extracts) was diluted 100-fold with PBST–BSA 0.1% to minimise the influence of the solvents. The diluted extracts were immediately assayed.

Baby food solid samples were spiked with known amounts of Zea. Subsamples of 1 g were weighed and transferred to a centrifuge tube and directly spiked with a stock solution of Zea in acetonitrile to final concentrations of 20 and 100 μg kg^{-1}. The samples were allowed to equilibrate overnight before extraction following procedure A.

For the liquid samples, the matrix effect was evaluated by preparing a standard curve for undiluted samples which were spiked with increasing concentrations of Zea (0–1000 μg L^{-1}). Similarly, a recovery experiment was performed on different cereal milkshake samples spiked with Zea within the range of concentrations used for the calibration curve. All of the experiments were analyzed in triplicate.

3. Results and discussion

3.1. Immunoassay optimisation

The concentrations of a specific antibody and the tracer are the key parameters that affect the sensitivity of competitive immunoassays. Typically, low antibody and enzyme tracer concentrations render high sensitivity; however, they must be significant enough to yield a measurable signal. In this paper, the coupling of specific antibodies to magnetic beads was achieved through the affinity of protein-G for the Fc region of antibody molecules. Proper orientation of the antibody binding sites is thus achieved, improving sensitivity. Since the amount of antibody available is directly related to the number of protein-G modified beads, their quantity could influence performance analysis. Different volumes of previously washed magnetic beads in suspension (0.5–8 μL) transferred to the wells were exposed to the immunoassay procedure with both a fixed excess of the other immunoreagents and a fixed incubation time (Ab: 20 μg mL^{-1}; tracer: 1:50 fold dilution; 120 min incubation times). As was expected, an increase in signal was observed with an increase in the volume of magnetic beads until the antibody concentration is the limited reagent (4 μL) (data not shown).

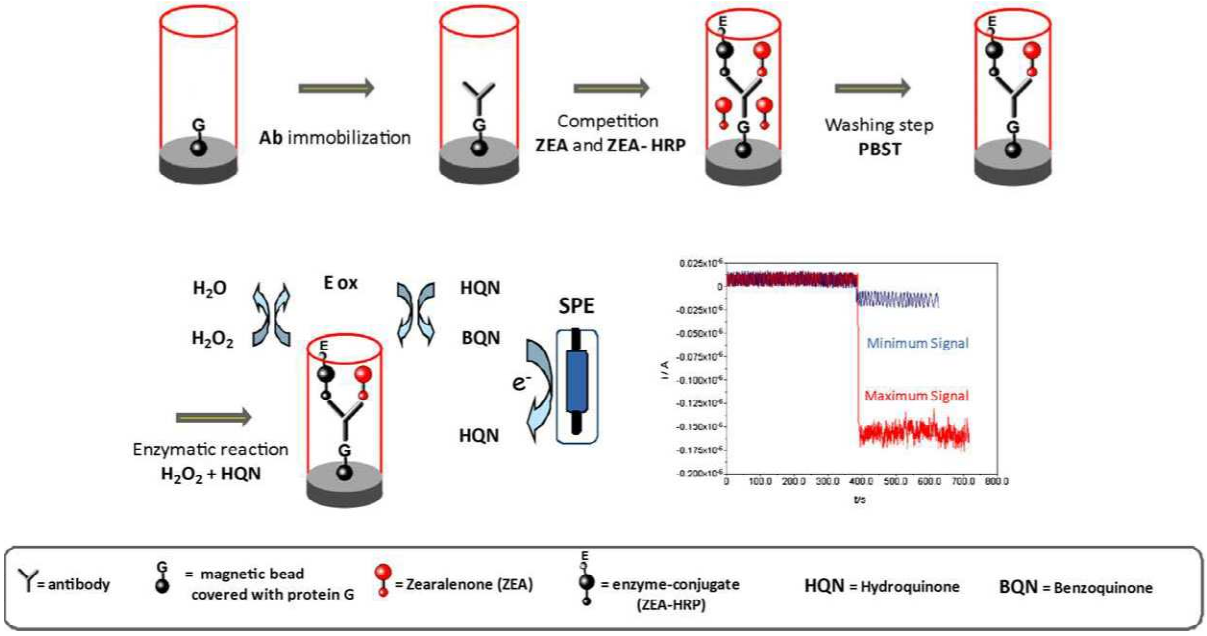

Fig. 1. Detailed schematic representation of the electrochemical immunoassay strategy for the detection of zearalenone.

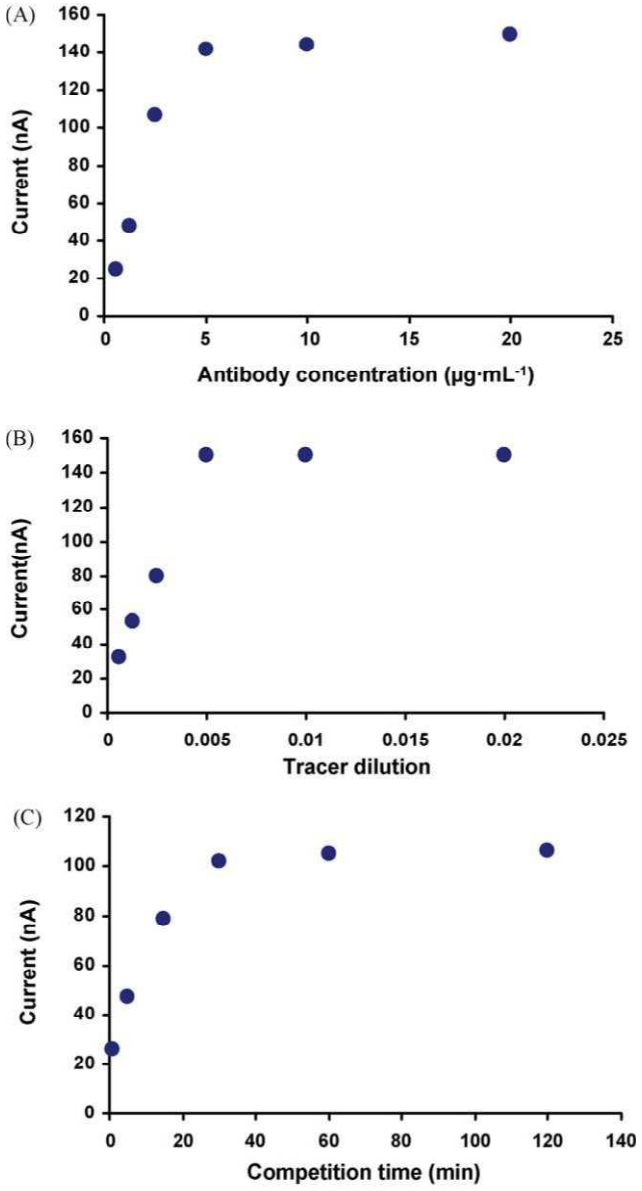

Fig. 2. Immunoassay parameters optimisation. (A) Optimisation of the antibody concentration. Current signals detected for different antibody concentration solutions when a volume of 2 μL magnetic beads and a fixed excess of the enzyme conjugate and incubation times were used. (B) Optimisation of the tracer dilution. The beads were incubated for 30 min with a 5 μg mL^{-1} of antibody solution. The incubation time with the tracer solution was 120 min. (C) Different incubation times between the antibody-coated beads and the tracer solution (1:200 with respect to the stock solution) were tested while keeping the other parameter as previously optimised.

For subsequent experiments, 2 μL of magnetic protein-G coated beads were chosen as a compromise in which a sufficiently high signal could be obtained while consuming the smallest amount of the expensive reagents.

In order to obtain the optimal concentration of antibodies added to the 2 μL of magnetic protein-G modified beads in the coating step, the dilution of the specific antibody was established by titrating a fixed amount of the tracer (dilution 1:50 with respect to the stock solution without competing with free Zea) with increasing concentrations of the antibody (0.62–20 μg mL^{-1}) (see Fig. 2A). A concentration of 5 μg mL^{-1} of monoclonal anti-Zea IgG saturated most of the protein-G coating sites available, hence it was used for the immunoassay procedure. Another parameter to optimise was the antibody immobilisation time onto the magnetic beads. Contact times ranging from 2 to 120 min were tested (data not shown). An increase in signal was observed up to 30 min after reaction at which point the current became constant. Hence, this was chosen as the minimum time to bind the antibody to the magnetic beads. Antibody-coated particles could be prepared in advance and kept at 4 °C for at least 2 weeks without activity loss.

An important point in competitive assays is that the amount of enzyme tracer dilution has to be limited in order to saturate the binding sites of the antibodies that are immobilised onto the solid phase. The antibody-modified magnetic beads were titrated with different tracer dilutions ranging from 1:50 to 1:1600 with respect to the stock solution. The results reported in Fig. 2B show the typical behaviour of a binding curve where the current values increased up to the dilution mark of 1:200, later reaching a steady state indicating the saturation of the antibody binding sites. Similarly, another parameter to optimise was the incubation time with the tracer or the competition time. These experiments were performed by incubating the antibody-coated magnetic beads with the tracer solution (1:200) for different periods of time (2–120 min). In Fig. 2C, it can be observed how the current signal increased until reaching a constant value after 30 min. This mark was chosen as the minimum time necessary for the affinity reaction.

The detection of the enzyme tracer was carried out at $E = -0.250$ V using H_2O_2 and HQN as the substrate and the electrochemical mediator, respectively. To optimise detection, concentrations of H_2O_2 and HQN were assessed for maximal amperometric current intensity. When H_2O_2 concentrations were evaluated within the range of 50–800 μM, maximal signal was achieved at 600 μM (data not shown). Higher concentrations yielded a decrease in the current intensity, probably due to the inhibition effect of an excess of H_2O_2. When concentrations of HQN were tested between 100 and 1000 μM, the current signal increased with concentration up to a value of 800 μM, after which it remained constant (data not shown). Therefore 600 μM H_2O_2 and 800 μM HQN were selected for further measurements. The incubation time of the enzyme substrate with the tracer also influenced the analytical signal. In principle, larger values render higher intensity currents up to the assayed time of 60 min. However, the mark of 25 min was selected as a compromise between optimal response and minimal analysis time.

Non-specific adsorption due to the direct adsorption of the enzyme tracer onto the functionalised magnetic beads or the vial walls can produce a residual background signal. This effect was evaluated by comparing the signals obtained in the immunoassay procedures with either antibody-coated or uncoated magnetic particles incubated with either only the tracer solution or the tracer solution and the excess of Zea mycotoxin (1 ppm). In both cases, in absence of the antibody and when elevated amount of competitive mycotoxin was present, the signals obtained were over 60% of the signal produced by the interaction between antibody and only tracer. When simply the enzyme tracer was added to the 1 mL glass vial where the immunoassay was performed, 38% of the affinity signal was obtained. This means that significant non-specific adsorption had taken place on the glass material itself, although previously blocked with a solution of PBST–BSA (1%). Therefore, for further experiments, single ELISA wells which had been previously blocked with PBST–BSA (1%) were chosen as containers. Under these experimental conditions, a reduction in background signal (excess of Zea) of up to 19% of the affinity signal (without competition with analyte) was obtained.

3.2. Immunoassay evaluation

The target analytical characteristics were LOD and the accuracy. LOD is a very important concern since future legislation will probably be stricter, especially for baby food.

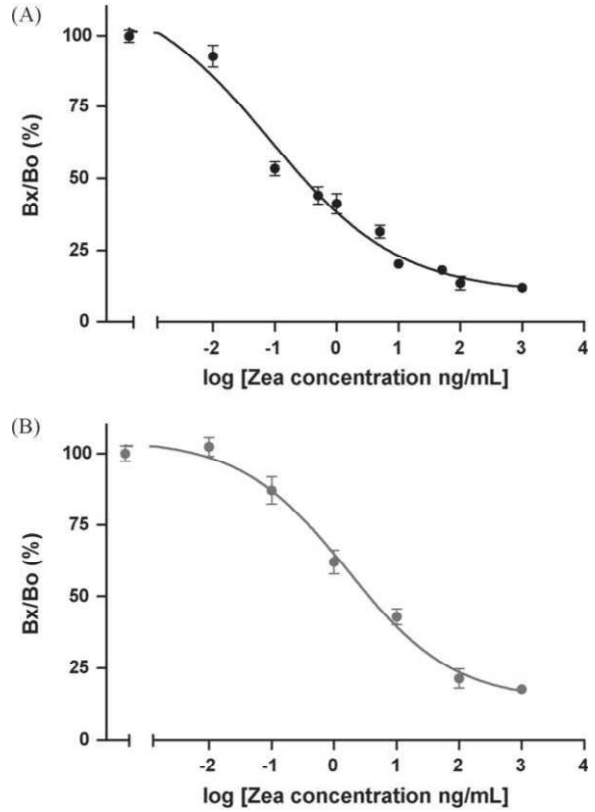

Fig. 3. (A) Calibration curve for zearalenone obtained using the competitive electrochemical immunoassay. (B) Calibration curve for zearalenone in undiluted spiked cereal milkshake samples. In both cases the points correspond to the *Bx*/*Bo* percentage ± SD, calculated for $n = 4$ repetitions.

Using the optimised parameters (beads volume: 2 μL; Ab concentration: 5 μg mL^{-1}; Ab incubation time: 30 min; tracer dilution: 1:200; tracer incubation time: 30 min; substrates concentration: 600 μM H_2O_2, 800 μM HQN; substrate incubation time: 25 min), the competitive calibration curves were performed by varying the amount of Zea from 0 to 1000 μg L^{-1} in PBST–BSA 0.1% buffer solution. Fig. 3A shows the competition curve for Zea where the signal is reported as the binding percentage *Bx*/*Bo* (%) versus a logarithm of the Zea concentration. *Bo* is the maximum signal obtained without competition with the tracer, and *Bx* is the signal obtained during the competition process. The curve exhibited the typical sigmoidal shape of a competitive immunoassay. The excellent detection limit, obtained as 85% of *Bo* (the maximal amperometric signal), was 0.011 μg L^{-1}. The EC_{50} value that corresponds to the analyte concentration necessary to displace 50% of the enzyme tracer was evaluated to be 0.079 μg L^{-1}. Intra-laboratory reproducibility was evaluated over four calibration curves on four different days in the concentration range of 0–1000 μg L^{-1}, and the relative standard deviation (RSD) average was calculated as 7.8%.

In order to assess the accuracy of the method, the analysis of a maize certified reference material (83 ± 9 μg kg^{-1}) was carefully carried out. Two different combinations of solvents in the extraction procedure were assayed. In procedure A (taken from literature), acetonitrile:methanol (50:50, v/v) was used as the extraction solution, while procedure B (taken from the CRM protocol) called for the use of acetonitrile:water (75:25, v/v). Table 1 list the levels found for Zea in the CRM analyzed using both solvent mixtures (three different subsamples each). In order to evaluate the results, one-sample *t*-test was executed. The results indicated that no differences were found at significance level of 0.05 indicating an excellent agreement between the Zea levels obtained by electrochemical immunoassay and the certified reference material.

Table 1
Analysis of a certified reference material (83 ± 9 μg kg^{-1})[a].

Extraction procedure	Found concentration (μg kg^{-1})	Recovery (%)
A	90 ± 4	108 ± 5
B	79 ± 5	95 ± 6

[a] Values are expressed as mean value ± standard deviation, $n = 3$.

3.3. Determination of zearalenone in baby food samples

Food samples with major analytical concerns such as prepared baby food containing different cereals were also analyzed by the immunoassay developed. This food matrix previously found to contain undetectable levels of mycotoxin, was afterwards spiked at two different concentrations of Zea, 100 and 20 μg kg^{-1}, the latter being the highest level allowed by the most restrictive legislation currently in force. After extraction according to procedure A, the previously centrifuged supernatant was diluted 100-fold in PBST–BSA 0.1% to avoid the matrix effect. It is important to note that the final theoretical Zea concentration in the extracted and diluted solution corresponding to the lower spiked matrix is 0.05 μg L^{-1}. This value is near the detection limit obtained in the standard calibration curve. Table 2 shows agreement between the added and found concentration for both spiked levels of the mycotoxin, which demonstrates the suitability of the method even at extremely low concentrations.

As was mentioned in Section 1, one of the main advantages of immunoassay techniques in general, and particularly with the strategy described in this paper, is their selectivity and simplicity. One of the problems of immunoassays, however, is matrix interference due to the presence of organic solvent solutions, lipids, vitamins, or proteins in the sample or the extracts that can disrupt the interaction between antigen and antibody [50,57]. Cleanup or dilution of the samples and extracts are the approaches most often used. In this paper, and for liquid samples such as cereal milkshakes, determination of mycotoxins was performed with no pre-treatment. In order to evaluate the matrix effects, the standard curve was prepared directly in undiluted cereal milkshake with increasing concentrations of Zea (0–1000 μg L^{-1}) (Fig. 3B) and compared with the standard curve prepared in buffer (Fig. 3A). It can be observed that both the EC_{50} values (0.079 μg L^{-1} in buffer vs. 1.5 μg L^{-1} in cereal milkshakes) and the detection limit (0.011 μg L^{-1} in buffer vs. 0.12 μg L^{-1} in cereal milkshakes) were slightly different. The accuracy of the electrochemical immunoassay to determine the concentration of mycotoxin directly in the raw liquid milkshake sample was studied in three levels of spiked samples at concentrations of 1, 10, and 100 μg L^{-1} of Zea. Using the previously obtained calibration curve (Fig. 3B) prepared in the blank matrix, recovery values in the range of 96–104% were reached, showing that the results obtained are very close to the spiked values as it is listed in Table 3.

Table 2
Recovery percentage for a powdered baby food containing a mixture of different cereals and spiked at two concentration levels[a].

Spiked concentration (μg kg^{-1})	Found concentration (μg kg^{-1})	Recovery (%)
100	108 ± 9	108 ± 9
20	28 ± 4	140 ± 20

[a] Values are expressed as mean value ± standard deviation, $n = 3$.

Table 3
Recovery percentage for a prepared liquid baby food (cereal milkshake) spiked at different concentration levels[a].

Spiked concentration ($\mu g\,L^{-1}$)	Found concentration ($\mu g\,L^{-1}$)	Recovery (%)
100	104 ± 11	104 ± 11
10	9.6 ± 1.3	96 ± 13
1	0.98 ± 0.16	98 ± 16

[a] Values are expressed as mean value ± standard deviation, $n = 3$.

4. Conclusion

Electrochemical detection coupled with immunoassay based on magnetic particles has demonstrated to be a very valuable tool for the ultrasensitive detection of zearalenone in baby food. This performance makes electrochemical immunoassays a very promising and timely analytical tool for future challenges in the area of food safety.

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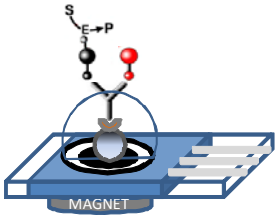

Desarrollo de un inmunosensor electroquímico, con calibración simplificada y haciendo uso de electrodos serigrafiados de carbono desechables, para la determinación de ZEA en alimentos infantiles.

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Simplified calibration and analysis on screen-printed disposable platforms for electrochemical magnetic bead-based inmunosensing of zearalenone in baby food samples

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ABSTRACT

An electrochemical immunosensor involving magnetic beads and disposable carbon screen-printed electrodes (SPEs) for zearalenone (Zea) sensing has been developed and evaluated using a certified reference material (CRM) and selected baby food samples. After the immunochemical reaction, the modified paramagnetic beads were confined by a magnet on surface of SPE platforms where electrochemical detection is simply achieved through the suitable substrate and mediator for the HRP enzyme. A remarkable detection limit of 0.007 $\mu g\,L^{-1}$ and excellent accuracy with recovery rate of 101–111% showed the proposed system to be a very suitable screening tool for the analysis of zearalenone in baby food samples.

A new simple, fast and reliable strategy involving the sequential performing of calibration and analysis of target mycotoxin using just one disposable SPE each is additionally proposed. Excellent analytical performance in terms of accuracy and precision were again obtained with a remarkable low systematic error (less than 4%) and excellent reproducibility (RSD = 6%). This strategy enhanced the analytical merits of immunosensor approach towards truly analytical disposable tools for food-safety scene.

1. Introduction

Zearalenone (Zea) is a nonsteroidal oestrogenic mycotoxin produced by several species of *Fusarium* (Weidenborner, 2001; Bennett and Klich, 2003). It can be found upon storage of different cereals such as corn, barley, oats, wheat, rice and sorghum, under high humidity and temperature conditions in temperate regions around the world (Betina, 1989). It has been reported in food or feedstuff containing the grain and it does not degrade at high temperatures (Kuiper-Goodma et al., 1987). Zea causes alterations in the reproductive tract of laboratory and domestic animals and in humans it has been associated with early puberty, hyperplasic and neoplasic endometrium and human cervical cancer (Zinedine et al., 2007; Ingle and Martin, 1986; Kuiper et al., 1998). Although Zea has relatively low acute toxicity, the European Union has recently set out the maximum levels in foodstuffs of *Fusarium* mycotoxins. 20, 75 and 100 $\mu g\,kg^{-1}$ are the highest allowed levels of Zea in baby food, cereal flour and unprocessed cereals, respectively (Commission Regulation No. 856/2005, 2005; Commission Regulation No. 1881/2006, 2006; Commission Regulation No. 1126/2007, 2007).

Due to the potential impact on human and animal health as well as from economical aspects, a broad amount of research has been produced to develop sensitive and specific methods for rapid and economic Zea detection in food, animal feed and complex biological samples (Krska et al., 2007, 2008; Krska and Molinelli, 2007). The most common analytical techniques applied for Zea analysis include thin layer chromatography (TLC) (Shumacher and Krska, 2001; Schaafsma et al., 1998); gas chromatography–mass spectrometry (GC–MS) (Tanaka et al., 2000); high-performance liquid chromatography (HPLC) using different detection principles as UV diode array (Briones et al., 2007; Zougagh and Ríos, 2008), mass spectrometry (Rosenberg et al., 1998; Zollner et al., 1999; Pallorini and von Holst, 2003; Berthiller et al., 2005; Cavaliere et al., 2005; Songsermsakul et al., 2006; Tanaka et al., 2006), fluorescence (Silva and Vargas, 2001; Radová et al., 2001; Mateo et al., 2002; De Saeger et al., 2003; Urraca et al., 2004; Reza et al., 2005; MacDonald et al., 2005; Schollenberger et al., 2006) as well as electrochemical detection (Andrés et al., 2008); and capillary electrophoresis (CE) (Maragos and Appell, 2007). These methods require expensive equipment, complicated and time consuming cleanup procedures and skilled operators that make them unsuitable methods for monitoring purposes.

Immunoassays, mainly ELISA methods, have proven to be an excellent technology as sensitive, specific, without the need of extensive cleanup, rapid, simple and with a high throughput for the detection of different chemicals in a wide variety of

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food matrices including mycotoxin analysis (Prieto-Simón et al., 2007; Goryacheva et al., 2007). Zea has been analyzed by different immunoanalytical methods including ELISA (Liu et al., 1985; Warner et al., 1986; Barna-Vetró et al., 1994; Bennet et al., 1994; Tanaka et al., 1995; Nuryono et al., 2005; Suzuki et al., 2007; Wang et al., 2008; Thongrussamee et al., 2008), fluorescence polarization immunoassay (Maragos and Kim, 2004), dipstick immunoassays (Schneider et al., 1995; Kolosova et al., 2007; Van Egmond et al., 2007), and flow-through immunosensor (Urraca et al., 2005).

Electrochemical immunosensors are receiving special focus in modern analysis because of their technical simplicity, sensitivity, low cost and possibility of decentralization in field analysis (Zacco et al., 2006). However, the use of the electrode surface as a solid phase for antibody immobilization as well as electrochemical transducer may result in a reduced electrochemical signal. The shielding of the electrode surface by the specific deposition of the antibodies can cause hindrance of the electron transfer. An alternative approach to improve sensitivity involves the use of electrodes for the transduction step, whereas the affinity reaction is performed in a different support as magnetic beads (Centi et al., 2007). On the other hand, magnetic beads have proven to be a powerful and versatile tool to improve the performance of immunoassays for food contaminants (Zacco et al., 2006, 2007; Centi et al., 2007; Gessler et al., 2006; Font et al., 2008). It has been reported that the use of immunobeads improves the performance of the immunological reaction due to an increase in the surface area, as well as the assay kinetics are achieved more rapidly since the beads are in suspension and the probability that antibody-coated magnetic beads meet the analyte is very high while keeping the solution under stirring. Furthermore, the magnetic beads can easily be manipulated through the use of permanent magnets, and the matrix effect is also minimized due to improved washing and separation steps which allows the analysis to be made without any pre-enrichment, purification, or pre-treatment steps. Direct electroanalytical detection of Zea has also been studied (Ramírez et al., 2005; Zougahg et al., 2008), although the sensitivity reached is not very high.

This work explores the use of disposable inmunosensing surfaces on screen-printed electrodes using magnetic beads coupled to ELISA method for the determination of zearalenone in different food matrices. In order to obtain a simple and disposable analytical tool for fast and reliable screening, the immunosensor was thoroughly validated towards the determination of zearalenone in a maize certified reference material using a strategy based on the sequential performing of simplified calibration and analysis protocols on the screen-printed immunosensing surfaces (SPIS).

2. Materials and methods

2.1. Chemicals and immunochemicals

Zearalenone (Zea), bovine serum albumin (BSA), hydrogen peroxide (30%), hydroquinone (HQ), benzoquinone (BQN) and Tween 20 were purchased from Sigma–Aldrich. Standard Zea solutions were prepared daily by dilution of stock solutions (5 mg mL^{-1} in acetonitrile). All the other reagents were of the highest available grade. Monoclonal antibody anti-zearalenone and the enzyme tracer Zea conjugated to HRP were supplied by Soft Flow Biotechnology (Gödöllõ, Hungary).

All buffer solutions were prepared with MilliQ water. Unless otherwise indicated, PBS or PBS modified with Tween and BSA was used. The composition of the PBS solution was phosphate buffer 10 mmol L^{-1}, pH 7.5, with 0.8% (w/v) NaCl, while in PBST solution 0.05% (v/v) Tween was also added. Citrate–phosphate buffer (24.5 mmol L^{-1} citric acid and 51.7 mmol L^{-1} dibasic sodium phosphate) pH 5.0, was used for washing magnetic beads and the antibody capture procedure according to the manufacturer's protocol.

2.2. Materials

Superparamagnetic polymer beads with protein G covalently coupled to the surface (Dynabeads® Protein G) were supplied by Invitrogen Dynal (Oslo, Norway).

Screen-printed electrodes (DS 110) based on carbon working and counter electrodes, and silver pseudo-reference electrodes were provided by Dropsens (Oviedo, Spain). The working electrode diameter was 4 mm.

2.3. Samples

Zea in maize certified reference material (83 ± 9 μg kg^{-1}) (ERM® BC717) was purchased from IRMM. Baby food cereal (Blevit multicereals) containing a mixture of wheat, rice, maize, barley, oats, sorghum, rye and soya flour was purchased from a local pharmacy (Madrid, Spain). Cereal milkshakes (Puleva) containing a mixture of wheat, rice, maize, barley, oats, and rye were purchased from a local retail store (Madrid, Spain).

2.4. Equipment

Electrochemical measurements were performed with a small portable potentiostat *μStat 100* from Dropsens (Oviedo, Spain) using a specific connector that acts as an interface between the screen-printed electrode and the potentiostat.

Magnetic separation during the washing steps was performed using a magnetic separation rack (Ohmicron). An ultrasonic bath from Sonics Vibracell was used in the extraction procedure.

The competitive curves were analyzed with a four parameter logistic equation using the proper software Graph Pad Prism 5.

2.5. Immunoassay scheme

The present immunosensing strategy is developed on the basis of a competition scheme where the mycotoxin zearalenone and an enzyme-labeled derivative compete for the binding sites of the specific antibody. Protein G covalently bound to magnetic particles acts as an oriented immobilization support for the capture of the anti-mycotoxin antibody. After molecular recognition, the extent of affinity reaction is evaluated by the addition of the enzymatic substrate and electrochemical mediator whose reduction on the electrode surface is directly related to the activity of the enzyme tracer (HRP-zearalenone) (Fig. 1).

Firstly, magnetic beads coupled with protein G were washed with citrate–phosphate buffer, pH 5.0, according to the manufacturer protocol in order to eliminate storage buffer and condition them for the IgG capture procedure. A volume of 2 μL of the beads was introduced in the ELISA well and then, 50 μL of specific antibody (5 μg mL^{-1}) prepared in citrate–phosphate buffer, pH 5.0 was added to the volume of Dynabeads. The solution was stirred gently for 30 min at room temperature to obtain antibody-modified beads. Washing steps (five times) with 200 μL of PBST allows elimination of not bound antibody. After each incubation or washing step, magnetic beads were separated from the supernatant by placing the microplate wells in a magnetic rack until magnetic beads migrated to the side walls and the liquid was clear to remove it.

The competitive assay was performed re-suspending the anti-mycotoxin antibody-coated beads in 50 μL of a mixture of zearalenone standard solution or sample and the enzyme tracer (final dilution 1:200 in PBST–BSA 0.1%). Competitive immunological reaction was allowed to proceed with gentle stirring for 30 min

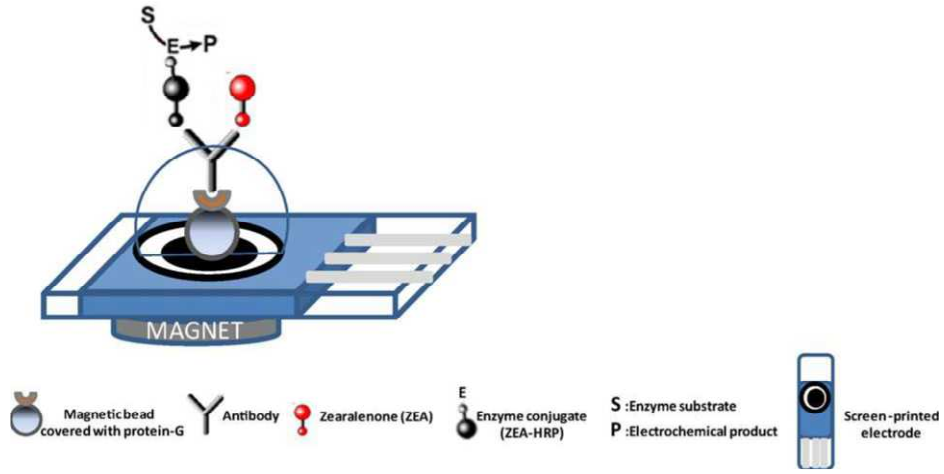

Fig. 1. Schematic representation of the electrochemical immunosensor strategy for the detection of zearalenone.

at room temperature. Magnetic beads were washed again with 200 μL of PBST five times to eliminate not bound species and finally re-suspended in 30 μL of PBS buffer. Once the affinity reaction took place and in order to perform the electrochemical transduction, just a 30 μL of suspension was transferred onto the surface of the screen-printed working electrode where magnetic beads are immobilized by placing the magnet on the bottom part of the electrode. This set up allows beads to be kept on the working electrode surface by avoiding the use of any additional cell. After that, 10 μL of a solution containing the enzymatic substrate (hydrogen peroxide, 80 μM) and the electrochemical mediator (hydroquinone, 40 μM) in PBS buffer was deposited on the electrode surface reaching enough volume for covering the three-electrode system (final volume 40 μL). After 5 min of incubation, the current response was measured using DPV. All DPV measurements were performed in the potential range 0.2 to −0.3 V, with a modulation time of 70 ms, pulse amplitude of 70 mV and a scan speed of 5 mV s^{-1}.

2.6. Calibration

The standard curve for Zea was fitted to a four parameter logistic equation using the software Graph Pad Prism 5 according to the equation:

$$Y = A + \frac{B - A}{1 + 10^{[\log\ EC_{50} - x]\ \text{Hill slope}}} \quad (1)$$

where A is the Y value at the bottom plateau of the curve (minimum intensity current corresponded to infinite concentration), B is the Y value at the top plateau of the curve (maximal intensity current corresponded to zero concentration), EC_{50} is the antigen concentration necessary to half the current signal, and Hill slope is the slope of the linear part of the curve.

2.7. Sample extraction procedure

Solid samples (1 g) were extracted with 4 mL of acetonitrile:water (75:25, v/v) for 90 min at room temperature in ultrasonic bath. Then, it was centrifuged at 4000 rpm for 10 min, extracted the supernatant and diluted 100-fold with PBST–BSA 0.1% to minimize the influence of the solvents. The diluted extracts were immediately assayed.

To assess accuracy, a maize certificate reference material (Zea level = 83 ± 9 μg kg^{-1}) was extracted under the previously commented method and assayed by the immunosensing procedure.

Baby food solid samples were spiked with known amounts of Zea. Subsamples of 1 g were weighed and transferred to a centrifuge tube and directly spiked with a stock solution of Zea in acetonitrile to a final concentration of 80 μg kg^{-1}. The samples were allowed to equilibrate overnight before extraction.

For the liquid samples (cereals milkshake) a recovery experiment was performed on undiluted samples spiked with a Zea concentration of 0.20 μg L^{-1}. All of experiments were analyzed in triplicate.

3. Results and discussion

3.1. Analytical performance of immunosensor approach for zearalenone determination

The immunosensing strategy developed is based on the use of protein G modified magnetic beads for a proper oriented immobilization of the specific antibody and a competitive assay where the enzyme tracer competes with the zearalenone for the binding sites of the antibody. Although sensitivity is determined by the affinity of the antibody for the specific antigen, improvements in detection limits can be obtained when appropriate assay conditions are chosen (Zacco et al., 2006). Different conditions such as the beads volume (2 μL), the antibody concentration used for the immobilization (5 μg mL^{-1}), the antibody immobilization time (30 min), the enzyme tracer dilution (1:200) and the competition time (30 min) were already optimized as reported in Hervás et al. (2009). Unlike the previous work, in this case, the immunoassay performance can be divided in two main steps which occurs at different places: (i) the affinity reaction, which takes place in solution (microtiter wells) with the aid of modified magnetic beads and (ii) the transduction step which occurs on the surface of screen-printed electrode. The affinity reaction process in solution entails the benefits of using magnetic beads such as higher surface area, higher probability of meeting the different immunospecies, and facility of manipulation. By other hand, the use of screen-printed electrode as electrochemical platform where magnetic beads (after immunoreactions) are entrapped for transduction event, improve sensitivity avoiding the shielding of the electrode surface by deposition of the specific antibodies, and then improving electron transfer. At the same time, this strategy diminishes the non-specific adsorption since affinity reaction is not performed on the electrode surface. Furthermore, the use of screen-printed electrodes allows the construction of a disposable immunosensor.

It must be remarked that the particular detection conditions where a drop localized on the surface of the SPE acts as

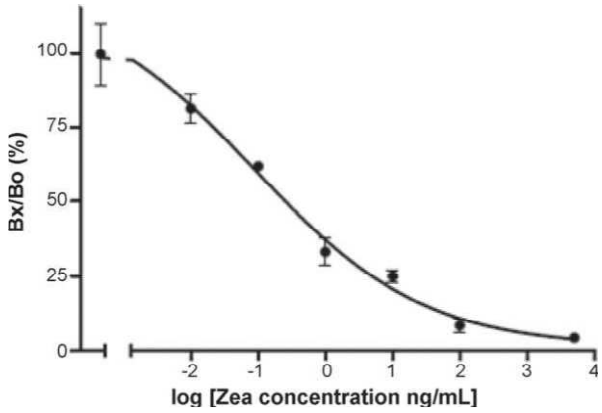

Fig. 2. Calibration curve for zearalenone determination using the competitive electrochemical immunosensor. The points correspond to the *Bx*/*Bo* percentage ± SD, calculated for $n = 4$ repetitions. (*Bo* is the maximum signal obtained without competition and *Bx* is the signal obtained during the competition process).

electrochemical cell. In this sense, re-optimization of the enzymatic substrate and mediator concentrations was needed. For this purpose, different hydrogen peroxide and hydroquinone concentrations were assayed after recognition event, where only tracer is added (0 Zea ppb). The greater electrochemical signal was achieved for 80 μM hydrogen peroxide and hydroquinone 40 μM (data not shown) which were chosen to perform the assays. Also, a control about the influence on magnetic beads on electrochemical behavior of hydroquinone was also tested indicating that the magnetic beads had not influence on the electrochemical response (see supporting information).

The competition curve for Zea, obtained in the conditions mentioned above and fitted accordingly to Eq. (1), is shown in Fig. 2. The signal is reported as a binding percentage *Bx*/*Bo* versus logarithm of the Zea concentration. *Bo* is the maximum signal obtained without competition and *Bx* is the signal obtained during the competition process. The limit of detection, calculated as 85% of the top value (maximal signal) was 0.007 μg L^{-1}. The IC_{50} calculated as the Zea concentration to produce a 50% decrease in the signal was evaluated to be 0.088 μg L^{-1}. The excellent limit of detection obtained assures a proper determination of the mycotoxin in real samples well below the legislative requirements.

To assess the accuracy of the immunosensor developed, a maize CRM having a concentration of 83 ± 9 μg kg^{-1} was analyzed. Three replicates of three maize CRMs independent portions were extracted according to the detailed experimental extraction procedure. After dilution 100-fold in PBST–BSA 0.1%, to avoid the solvent matrix effect, the samples were directly measured. The results are reported in Table 1 where recovery values ranging between 101% and 111% can be observed. In order to evaluate the results, one-sample *t*-test was executed. The results indicated that no differences were found at significance level of 0.05. The excellent agreement from the obtained results within the uncertainty of the CRM demonstrates the suitability of the immunosensor for the screening of real sample contamination.

Different food samples with major analytical concern such as a baby food powder and a liquid sample (cereals milkshake) containing mixtures of cereals were analyzed by the immunosensing strategy. The baby food powder, previously found to contain an undetectable level of the mycotoxin was afterwards spiked at a concentration of 80 μg kg^{-1}. Due to its solid nature, extraction and dilution was carried out according to the experimental procedure reaching the final theoretical concentration of 0.20 μg L^{-1}. Liquid samples were directly analyzed without any extraction procedure or dilution step to explore the presence of the mycotoxin. After the sample was found to contain an undetectable level of Zea, spiked samples at 0.20 μg L^{-1} (since this level was the Zea level really measured in the solid sample, well below the European directive requirements) were prepared and tested by the immunosensor.

Tables 2 and 3 show the recovery values obtained for both types of samples. Excellent agreement between the added concentration of Zea and the obtained results is achieved for both solid and liquid samples demonstrating the suitability of the immunosensing strategy for determination of the mycotoxin in baby food samples.

Table 1
Analysis of a certified reference material (Zea 83 ± 9 μg kg^{-1}).

Signal (μA)	Found concentration (μg kg^{-1})	Recovery (%)
0.595	88	106
0.590	92	111
0.603	84	101
	88 ± 4	106 ± 5

Table 2
Recovery percentage for a powdered baby food containing a mixture of different cereals and spiked at concentration level of 80 μg kg^{-1}.

Signal (μA)	Found concentration (μg kg^{-1})	Recovery (%)
1.045	84	105
1.066	78	97
1.058	80	100
		101 ± 4

3.2. Simplified calibration and analysis protocols on the disposable SPE platforms

In order to diminish the time consuming and laborious procedure of routinely performing a four parameters logistic calibration curve for sample analysis, an integrated calibration and analysis on board of the same disposable SPE was proposed and tested. The methodological strategy implies the sequential determination of a selected concentration of Zea with calibration purpose, followed by the analysis of the food sample. Comparison of both signals and after the adequate definition of the calibration factor, allows the determination of the mycotoxin in the sample. Indeed, the calibration factor is calculated as $f_{calibration} = S_{Zea\ standard} \times [Zea]_{standard}$. Since this calibration factor is a constant for each analysis, Zea concentration in the sample can be calculated as $[Zea]_{sample} = f_{calibration}/S_{Zea\ sample}$. This methodology is clearly advantageous because: it generates a simplification in the immunoanalysis calibration procedure; calibration and analysis are performed under equal conditions avoiding other sources of error; and the calibration step can be suitably designed using the same concentration for the control as the one expected in the sample, improving the precision (and subsequently the accuracy) of the analysis, usually with the signal in the linear range of the calibration graph where the error is minimized.

In order to demonstrate the suitability of the simplified calibration strategy, the Zea concentration was determined for 10 independent CRM samples using 1 different electrode each. Table 4 lists the results obtained using this strategy. Interestingly, all the values were found within the uncertainty given by the refer-

Table 3
Recovery percentage for a prepared liquid baby food (cereals milkshake) spiked at concentration level of 0.2 μg L^{-1}.

Signal (μA)	Found concentration (μg L^{-1})	Recovery (%)
0.813	0.197	98
0.820	0.193	96
0.813	0.197	98
		98 ± 1

Table 4
Analysis of a certified reference material (Zea 83 ± 9 μg kg^{-1}) using the sequential simplified calibration and analysis on board of different SPE platforms.

Electrode number	Calibration of Zea			Analysis of Zea in CRM		
	[Zea][a] (μg L^{-1})	$S_{standard}$[b] (μA)	Calibration factor, f[c] (μA μg L^{-1})	S_{CRM}[d] (μA)	[Zea][e] (μg L^{-1})	$[Zea]_{real}$[f] (μg L^{-1})
1	0.20	0.44	0.09	0.41	0.22	88
2	0.20	0.39	0.08	0.41	0.20	80
3	0.20	1.32	0.26	1.38	0.19	76
4	0.20	1.25	0.25	1.27	0.20	80
5	0.20	1.28	0.26	1.37	0.19	76
6	0.20	1.26	0.25	1.35	0.19	76
7	0.20	1.32	0.26	1.40	0.19	76
8	0.21	1.79	0.38	1.76	0.22	88
9	0.21	0.24	0.05	0.27	0.19	76
10	0.21	0.50	0.10	0.46	0.22	88
					Mean value ± s	80 ± 5

[a] Concentration of Zea standard used for calibration.
[b] Analytical signals obtained for the Zea concentration during calibration protocol.
[c] Calibration factor defined and calculated as $f_{calibration} = Signal_{Zea\ standard} \times [Zea]_{standard}$.
[d] Analytical signals obtained during the determination of Zea in the CRM.
[e] Concentration of Zea obtained in certified reference material using a calibration factor and calculated as: $[Zea]_{CRM} = f_{calibration}/Signal_{CRM}$.
[f] Real concentrations obtained in the CRM.

ence material, revealing a very good accuracy and demonstrating the suitability of the strategy. In addition, this approach creatively avoided the typical loss of reproducibility between SPEs because of calibration was performed for every analysis calculating suitably the factor each. Even, when the mean value was calculated taking account the 10 electrodes used, an excellent agreement (80 ± 5 mg kg^{-1}) with the reference value given by CRM (83 ± 9 mg kg^{-1}) was also obtained. This finding underlined the potency and utility of the proposal, giving a real alternative for reliable and disposable screening of mycotoxins.

4. Conclusion

An electrochemical immunosensor based on magnetic beads for high sensitivity detection and accurate determination of zearalenone in baby foods in creative connection with a novel simplified calibration and analysis strategy has been successfully developed. Remarkable limit of detection has been demonstrated accordingly to the actual requirements established for this mycotoxin in baby food containing cereals as well as an excellent accuracy. In addition, the excellent analytical performance found when calibration and analysis were carried out using just one SPE platform; added a valuable analytical merit and potency to the electrochemical immunosensor, opening new gates and become a very promising disposable analytical tool for food-safety diagnosis.

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The authors are very grateful to Soft Flow Biotechnology for kindly providing the monoclonal antibody and the enzyme tracer. Financial support from the Spanish Ministry of Science and Innovation CTQ2008-06730-C02-02/BQU (A.E.) is acknowledged. MHY acknowledges the fellowship received from the Spanish Ministry of Science and Innovation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.12.031.

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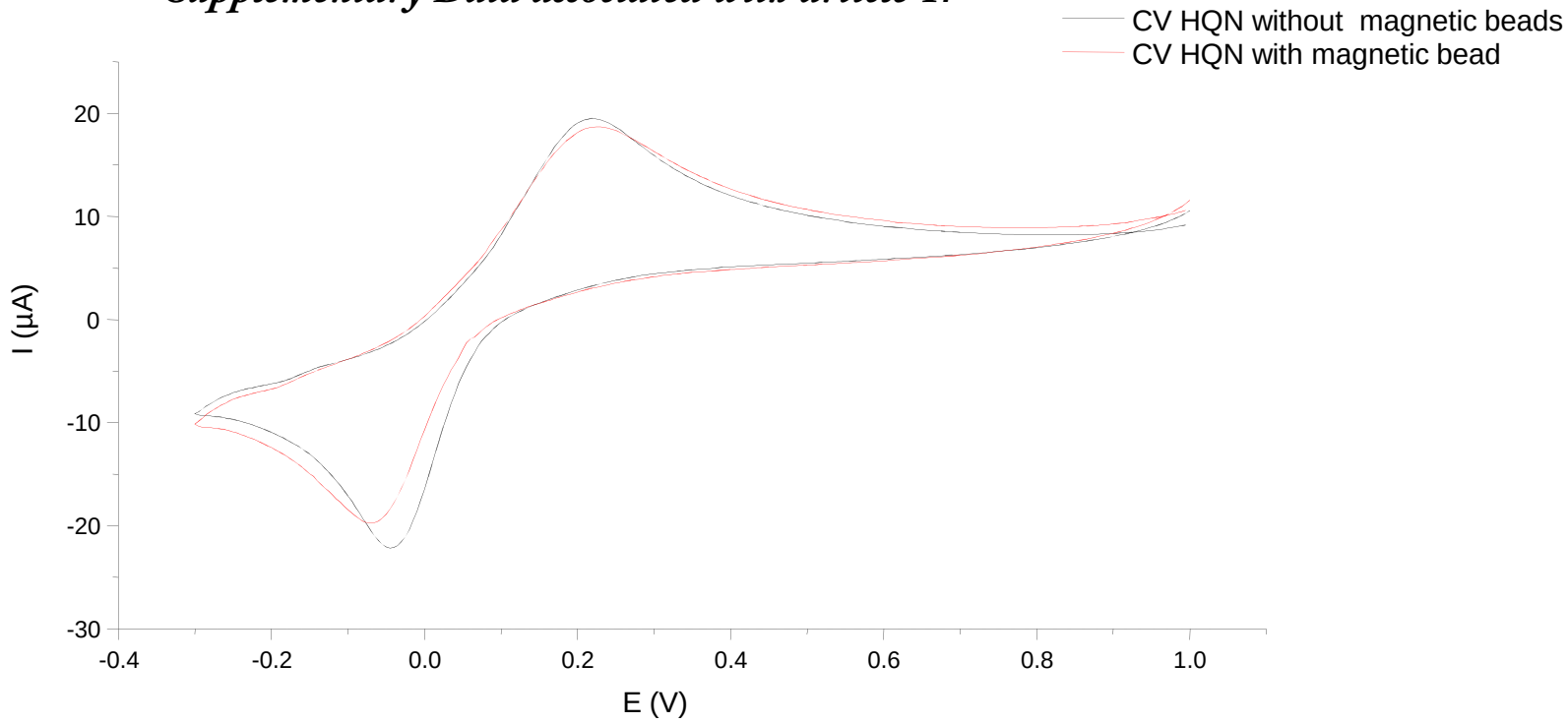
Supplementary Data associated with article 1.



CV HQN 800µM
(with magnetic beads)

$\Delta E = E_{ox} - E_{red}/ 2 = 0.215V - (-0.065V)/2 = 0.07V$

$I_{ox} = 18.1\ \mu A \qquad I_{red} = 15.3\ \mu A$

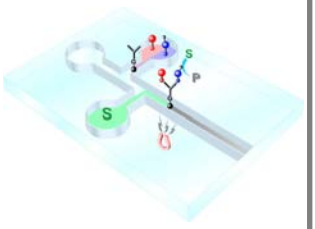
CV HQN 800µM
(without magnetic beads)

$\Delta E = E_{ox} - E_{red}/ 2 = 0.215V - (-0.04V)/2 = 0.08V$

$I_{ox} = 18.9\ \mu A \qquad I_{red} = 19.3\ \mu A$

Cyclic voltamperograms showing irrelevant effect corresponding to the applied magnetic field on the electrochemical behavior of the enzymatic generated redox mediator.

III.3 REVISIÓN BIBLIOGRÁFICA:



Inmunoanálisis electroquímico integrado en plataformas microfluídicas

ELECTROCHEMICAL IMMUNOSENSING ON BOARD OF MICROFLUIDIC CHIPS PLATFORMS

Mirian Hervás, Miguel A. López, Alberto Escarpa

Index

Abstract

Microfluidic devices as immunosensing platforms have attracted a great attention in the last years becoming an emergent technology in different areas as biomedical, pharmaceutical, environmental and food analysis. Combination of the remarkable features from microfluidic platforms together with those derived from immunoassays, produces a promising tool for selective, sensitive, automatic and point-of-care testing in real applications. In this article, different aspects related to microfluidic material substrate, fluid handling, multiplexing and mainly, the different surface modification and immunoreagents immobilization strategies, have been reviewed and discussed from 2005. Although different detection modes can be used in this kind of microfluidic immunosensing platforms, the special features of electrochemistry have made this technique widely expanded, being the common detection scheme for the articles reviewed. Finally, future perspectives on microfluidic immunosensing are provided.

1. Introduction

Microfluidic analytical systems, also referred as "Lab-on-a-chip" or "micro-total-analysis-system" (μTAS), include those systems capable of integrate, into a miniaturise device, all the analytical stages usually performed in a laboratory (sample pretreatment, sample/reagent transport, mixing, reaction, separation, detection). By other hand, microfluidic technology seeks to improve analytical performance by reducing the analysis time, decreasing the consumption of sample and reagents, diminishing risk of contamination, using lower power consumption, increasing reliability, functionality and sensitivity through automation, integrating multiplexing analysis, and specially portability providing the possibility of point-of-care applications. Although this methodology is often envisioned as miniaturised version of its large-scale counterpart, it has to be remarked that physical and chemical process could present different behaviours. This is due to micrometric dimensions of the channels and fluids volume in the nL and pL scale, since they are governed by the laminar flow what makes that fluidic streams flow parallel to each other and mixing occurs only by diffusion. Looking at the current analytical literature, is clear the great importance of this technology in the contemporary scientific scene.

Immunological methods make use of antibodies as analytical tool for detecting a plethora of clinical, environmental and food relevant analytes. The special features that had made immunoassay widely increased in the last decades are the highly sensitivity and specificity of the antibody-antigen interaction. Although enzyme-linked immunosorbent assay (ELISA), currently performed in microtiter plates is the most common technique, a variety of assay types can be performed depending of the analytes or samples. A fundamental classification differentiates between heterogeneous and homogeneous assays. In a heterogeneous format, antibodies (or sometimes antigens) are immobilised on a solid support where the complex is formed. However, in a homogenous format the antibodies and antigens freely form an immunocomplex in the solution phase. Although both types have been studied and can be easily implemented in microfluidic platforms, heterogeneous assays benefit from the high surface area/volume ratio that rend higher sensitivity while homogeneous format take advantage from the possibility of multiplexing format and fast electrophoretic separations. Immunoassays also can be classified as either a competitive or non-competitive format. In the competitive type, the analyte competes with a labelled analyte for a limited number of antibody binding sites. As the amount of analyte in the sample increases, a displacement of the labelled analyte bound to the antibody binding sites occurs. This fact produces a decrease in the detection signal if the complex antibody-labelled analyte is detected or a signal increase if the labelled free analyte is detected. Alternatively, in non-competitive immunoassays (e.g. sandwich format) the detection signal increases in proportion to the analyte concentration.

Combination of both techniques explodes the selectivity and sensitivity from immunoassays with the remarkable features stated above from microfluidic platforms. Microfluidic immunoassays make use of a network of microchannels and/or immunoreactor chambers usually built in a monolithic platform (microchip, no bigger than the size of a microscope slide) from different materials as silicon, glass or polymers with part of all the necessary components of an immunoassay procedure integrated. These microfluidic platforms are especially suitable for immunoassays from different points of view. Firstly, the long-time associated to incubation stages in normal immunoassay can be attributed to the inefficient mass transport for the immunoreagents to move from the bulk solution to the wall surface where interaction takes places. In microchannels, the surface area to volume ratio is higher which makes the diffusional

distances dramatically reduced and produce lower analysis times. Furthermore, the miniaturisation reduces drastically the consumption of the expensive reagents as well as those special samples (i.e. neonatal blood, spinal fluid). By other hand, automated procedure can be potentially performed since different steps and fluid movement can be easily controlled, especially with electrokinetic fluidic motivation, through the adequate control of applied electric fields, or in a more complicated way by the uses of pumps, valves, mixers, etc. The introduced advantages have made microfluidic immunoassay platforms as an emergent and powerful alternative in the analysis of a broad variety of analytes in different fields such as medical diagnosis, pharmaceutical and biological research, food industry and environmental applications. Consequently, numerous research achievements have already been reported in the last years, and some of them revised in excellent review articles [1-5].

From the beginning, detection has been one of the main challenges for analytical microsystems, since very sensitive techniques are needed as a consequence of the ultra-small sample volumes used in micron-sized environments. A collection of detection methods has been implemented as signal transduction pathway to recognise the antibody/antigen event in microfluidic immunosensors. Although label-free detection strategies are possible (surface plasmon resonance, quartz crystal microbalance, wavelength interrogated optical sensing, silicon nanowire field effect transistors, imaging ellipsometry), usually one of the immunospecies must be conjugated with specific labelling molecules to facilitate the detection of analytes. Current labels include fluorescent and redox molecules, together with enzymes. Enzymes as labels provide a great amplification of the signal and increase the sensitivity due to the catalytic turnover, which implies that the number of detectable molecules can be exponentially higher than the number of antigens. A wide variety of detection labelling alternatives can be used in microfluidic systems such as chemiluminiscence, static light scattering, magnetoelectronic, thermal lens microscope; but laser-induced fluorescence (LIF) and electrochemical detection (ED), primarily, along with mass spectrometry are the most commonly used routes. LIF was the original detection technique and it is the most often used detection scheme because of its inherent sensitivity. However, the high cost and large size of the instrumental set-up for LIF has been sometimes incompatible with the concept of μTAS. In addition, tedious derivatization schemes are needed to use LIF with non-fluorescent compounds. The principal alternative to LIF detection is ED.

Electrochemical detection has been proven as an ideal and valuable technique to be incorporated in miniaturised devices, due to its inherent facility for miniaturisation without loss of performance, high sensitivity and compatibility with microfabrication techniques. Likewise, its responses are not dependent on the optical path length or sample turbidity, and it has low power supply requirements, which are additional advantages. Either electroactive molecules, or enzymes producing redox molecules as catalytic product, can be used for labelling antibodies. From the arsenal of electrochemical techniques, generally amperometry is ideally suited for microfluidic immunosensors since reduction in dimensions of microelectrodes improves the sensitivity. This reduction in the size of the electrodes produces a shift from planar to non-planar diffusion leading to efficient collection of electroactive species. That effect results in an increase of the signal to noise ratio, which generally translates into a lower detection limit.

The scope of this review article is restricted to electrochemical immunoassays carried out in microfluidic platforms gathered from 2005 to 2011. Relevant scientific contributions will be presented according to different areas such as the material substrates and fluidic handling; while the antibody immobilisation strategy would be a key classification aspect, where different possibilities are listed: (i) direct immobilization onto the microchannel-wall surfaces, (ii) immobilization onto microbeads; (iii) immobilization onto electrode surfaces within the microchannels.

2. Materials

The material substrate used for microchip fabrication is a key factor since the performance of the microfluidic platform will be strongly dependant of this material. The immobilisation of the antibodies, the fluid handling and even detection must be designed according to the material chosen or vice versa. Silicon, glass and polymers are the three main types of materials used for microfluidic devices fabrication. Advantages and limitations are presented for each of these materials while some examples for microfluidic immunoassays using these materials will be brought from the literature.

Silicon was the first material used in microfluidic fabrication due to the well-established microfabrication processes developed for semi-conductor microelectronics industry.

Chemical and physical properties of silicon are well characterised: It possesses good thermal conductivity, it is resistant to high temperatures and structurally strong, can be produced virtually in any geometrical microstructure with high precision, and its surface chemistry has been studied extensively giving the capability to attach molecules to its surface easily. However, it present some important limitations such as being not optically transparent in the wavelength range for optical detection; it is electrically conductive, thus resulting unsuitable for electrochemical detection or electrokinetic flow transport of reagents; proteins and other biomolecules tend to bind to silicon surface groups, reducing the sensitivity (non-specific adsorption); the microchip construction is relatively expensive, time consuming and the wet etching process typically used in fabrication produces a low aspect ratio. Although silicon has been a significant material in microfluidic devices these drawbacks have made it replaced or combined with glass or polymer substrates for electrochemical immunoassay applications.

Besides, the use of glass as material substrate can overcome some of the problems associated to silicon. Glass is less expensive, it presents good chemical resistance and it is optically transparent through the visible spectrum allowing LIF and other optical approaches for detection. Furthermore, thanks to be none electrically conductive, glass is compatible with electrokinetic fluid transport and it is still highly exploited in capillary electrophoresis and immunoassay involving electrochemical detection. However, some drawbacks are also presented in this material. As in the case of silicon substrate, the wet etching method used for fabrication does not produce a high aspect ratio, toxic chemicals are involved, and this material is more fragile. Few examples of electrochemical microfluidic immunoassays fully developed in glass are reported in the literature. Tang et al. [6] have presented an arrayed immunoassay for electrochemical detection of four tumour markers, using a magnetic controlled microfluidic device produced in glass. By other hand, our group has recently reported a magnetic beads-based electrochemical immunoassay, where one of the beauties of the strategy is the use of a simple double-T geometry glass microchip for reliable control of permitted levels of zearalenone mycotoxin in infant foods [7].

In the last years, polymers have emerged as an interesting alternative to glass and silicon in the microfluidic immunoassay scene. Polymers offers the advantage of being low cost and easily produced since microfabrication techniques like injection molding,

compression molding, extrusion, hot embossing, soft lithography, laser photoablation, screen printing and replication are highly reproducible; while expensive patterning procedures are only required for constructing the mold. In addition, they present good chemical resistance, optical transparency, low auto fluorescence, none toxic and absence of UV absorption. Moreover, a variety of surface modification methods are available for immobilization of different antibodies or antigens, improving the efficiency of these devices. There are a variety of materials such as plexiglas, cyclic olefin copolymers, polyester, polycarbonate, polyimide, polymethyl methacrylate (PMMA), each with its specific properties offering the possibility of tailoring a material to a specific application. However, without any doubt by the number of reported articles, poly(dimethylsiloxane) (PDMS) have emerged as the most used polymer material for microfluidic immunoassays. PDMS is flexible, optically transparent, impermeable to water and permeable to gases, and very compatible with biological studies. Nevertheless due to its hydrophobic surface, this material presents some limitations as poor wettability, forming bubbles in aqueous solutions, lower durability and the most transcendental for immunoassays is the tendency to adsorb proteins and other molecules, rending a high nonspecific adsorption and hence producing an elevated background signal. However, and due to the elastomeric nature of PDMS that allows an easy seal with smooth and flat surfaces, hybrid devices using PDMS and other different type of material as glass, polyester have been reported. A summary of these devices is presented in **Table 1**.

Apart from these more established materials, a low-temperature co-fired ceramic (LTCC) has also been used for constructing microchannel devices for electrochemical microfluidic immunoassay. Using this material, a highly intrincated three-dimensional structures integrating fluid networks, embedded passives and electronic circuits by means of unfired flexible sheets can be designed. After a process of firing, the LTCC becomes rigid with very good thermal, mechanical and electrical properties [8].

3. Fluid delivery

The liquid transport system inside a microchannel plays an important role in microfluidic immunoassays since its performance directly affect the results of the

assays. Different strategies for fluid flow into microfluidic immunoassay devices have been reported in the literature, mainly categorized as pressure, electric and passive fluid handling forces.

Pressure-driven flow is the most widely used mode of fluid delivery for microfluidic immunoassays, even with electrochemical detection. Buffers and reagents can be delivered inside the microfluidic system generally with the aid of syringe micropump or, lower extended, by applying vacuum at the outlet of the channel. This modality is compatible with a wide range of substrate materials and solvent composition, including the non-electrically conductive fluids. However, irreversible sealing is usually necessary due to the pressure experienced by the device and by other hand, pressure-driven flow has a parabolic velocity flow profile, which causes sample plug dispersion and peak broadening, rendering it less attractive for separations. Also micropumps like peristaltic pumps are well suited, although the flow behaviour is usually non-linear that may be a problem for this kind of devices. Most of the articles gathered in this review deal with pressure-driven flow by using a syringe pump (see **Table 1**). However, Park et al. [9,10] have developed lab-on-a-chip devices, where the hydraulic pressure caused by the elastic deformation of the liquid reservoir can be used as the driving force for transfer multiple liquids into the device. This is achieved using elastic PDMS cover caps with a reversed mushroom-shaped locking component to retain the internal pressure, which acts as the latch. In this case, two liquid reservoirs with the latch are integrated in the LOC for sophisticated fluidics, according to the sensing strategy (**Fig. 1**).

Electric forces as a fluid transport system relays on the flow generated by electrophoretic and electroosmotics interactions of applied electrical fields with ionic species in solution. In electrophoretic flow, charged molecules are manipulated in the presence of electric field by electrostatic forces. Electroosmotic flow is the bulk flow resulting from the effect of the electric field on the solution double layer at the channel wall. A layer of fluid enriched in solvated ions (typically, cations) is attracted to the oppositely charged walls to maintain charge balance. In the presence of electric field, the solvated ions and their water of hydration are drive toward the oppositely charged electrode while dragging the bulk fluid via viscous forces to form a uniform plug-like flow. Using this mechanism, the steps for flow switching, sequencing, and stopping can be easily achieved by controlling the applied electric field. Applying appropriate voltage polarity to the channel reservoirs and/or changing the net charges on the channel

surface can control even the direction of the flow. Therefore, electrokinetically driven flow is a well-established fluid actuation in micro total analysis systems that are amenable of automation. Especially suitable for homogeneous microfluidic immunoassays whit CE separations and electrochemical detection [11], it has also been used for heterogeneous microfluidic immunoassays [7,12,13].

An emerging electric fluid manipulation called electrowetting force is a liquid transport strategy whereby liquid discrete droplets are manipulated in the presence of programmed voltage sequences applied to an electrode array. By applying sequences of AC or DC electric potentials between ground and actuation electrodes, droplets of reagents or samples can be driven to move, merge, split and dispense from reservoirs by a combination of electrostatic and dielectrophoresis forces. Nashida et al. [14] have developed an integrated microfluidic device where injecting, flushing, and sensing functions were realized using valves that operate based on direct electrowetting. The injection and flushing of solutions were controlled by gold working electrodes, which functioned as valves. The valves were formed either in the channels or in a through-hole in the glass-PDMS substrate. The simple structure and function facilitate the integration of many flow channels and the handling of many solutions. A partial simulation of a sandwich immunoassay for AFP determination demonstrated the system´s applicability.

Passive forces that not require external power sources to drive liquids have been also reported for electrochemical microfluidic immunoassays. In a commercial and highly automated design for microfluidic ELISA reported by Rossier et al. [15] only gravity and capillary forces for driving fluidics within the microchannels are used. The system comprises ("*immunochip*") with eight parallel microchannels etched into a polyimide substrate at distances compatible with conventional 96-well plate automation. A robotized platform dispenses samples and reagents from conventional plates or tubes into microchannels of the immunochip in which assays are processed and results readout. The immunocomplex formed between the samples, enzyme conjugate and antibody functionalized magnetic nanoparticles takes places in a reaction plate (either competitive or sandwich format). After incubation, the liquid handler dispenses the incubation mix in the top reservoir of the microchip. Each microchannel has a length of 1.5 cm and a total volume of 300 nL. The solution flows freely by gravity and capillary forces through the microchannels, while beads are trapped nearby the electrodes by virtue of a magnet array. The upper part of each channel contains a total of 42 gold

working electrodes all connected together. After dispensing the enzymatic substrate, electrochemical signal is measures on the electrode surfaces. Quantification of recombinant IgG in mammalian cell-culture aliquots was performed to demonstrate the suitability of the robotized microfluidic immunoassay system.

4. Microfluidic immunoassay configuration

Electrochemical microfluidics immunoassays can be conducted either in homogeneous and heterogeneous configurations. In homogeneous configuration, both unbound antibody and antigen-antibody complex are freely placed in the solution. Discrimination of both species in microfluidic devices is usually accomplished by their electrophoretic mobility differences in microchannels. In heterogeneous assays, either antibody or antigen are immobilised on a solid surface and a separation step is performed to isolate the desired analyte from any other potential interferences. Heterogenous immunoassays are predominantly used in microfluidics and different strategies and examples will be provided.

4.1 *Heterogeneous microfluidic immunoassays*

Immobilisation of antibodies onto solid supports is highly important since it greatly influences the sensitivity and reproducibility of the assays. Several strategies have been implemented, although they can be divided in those where antibodies are immobilised on the surfaces of microchannels, on microbeads, or on the electrode surfaces that usually are positioned within the microchannels.

4.1.1 Antibody immobilisation on the microchannels surface

One of the most attractive characteristics, and highly improving the conventional immunoassays performed in microwells, is the high surface-volume ratio of the miniaturised microfluidic devices by significantly diminishing the diffusion distance that leads to fast analysis time and more sensitivity assays. However, this property also enhances the interaction antigen/antibody with the surface material that may result poor protein activity and non-specific protein adsorption. Therefore, it is a mayor interest to control the surface properties to achieve accurate and reproducible analytical results. Generally, antibody immobilisation is performed in polymer microchannel walls from

PMMA or PDMS, which are hydrophobic, although more polar surfaces as silicon and glass can be rendered hydrophobic by silanization. The physical adsorption of antibodies on such surface may cause protein denaturation leading to poor protein activity, mainly because of unfavourable orientation, steric hindrance and denaturation. To get this surface protein compatible, chemical surface modification is accomplished through covalent bonding between specific biocompatible reagents and material surface. Furthermore, in some cases and by means of adequate linkers, oriented antibody immobilisation can be obtained. By other hand, it is important to remark that a further limitation of immobilisation on hydrophobic surfaces is that blocking steps are usually necessary to limit the non-specific binding of proteins and small molecules, which usually acts as a central issue in this assays.

Zhou et al. [8] performed the immobilisation of the specific antibodies against cardiac troponin I (cTnI) and C-reactive protein (CRP) on PDMS-gold nanoparticle (GNP) composite microreactors. GNPs are synthesised in situ on the native PDMS to form the PDMS-GNP composite where the antibodies are adsorbed. After the corresponding antibodies capture the antigens, quantum dot labeled secondary antibodies (CdTe-Ab1, ZnSe-Ab2) are combined with antigens. Detection of both antigens is carried out simultaneously by the measurement of the corresponding metals after the dissolution of QDs with stripping voltammetry in end-channel carbon fiber electrode. Low detection limits of 0.004 $\mu g\ L^{-1}$ for cTnI and 0.22 $\mu g\ L^{-1}$ for CRP were coupled with excellent selectivity and the absence of nonspecific adsorption effects. The stereo structures of gold nanoparticles provide a biocompatible interface unlike direct adsorption on PDMS, which enhances the capability of capturing the target protein, which may improve the detection sensitivity.

Jang et al. [16] have reported a microchip fabricated on a glass substrate with a three-electrode system integrated and assembled with a surface-functionalized PDMS where the antibody is immobilised (**Fig. 2**). The internal surface of the PDMS channel is chemically modified into a silane monolayer containing poly(ethyleneglycol) (PEG) groups and surface-exposed biotin molecules to immobilise biomolecules via avidin-biotin linkage. PEG reduces the negative effect of non-specific adsorption, while avidin is used as a linker between the biotin derived surface and the biotinylated capture antibody. A sandwich immunoassay format with (alkaline phosphatase) AP-labelled

secondary antibody is used to determine mouse IgG as a model analyte. A rapid immunoassay (8 min for total assay time) whit a low detection limit (485 pg mL^{-1}) and 95% confidence level is achieved. Additionally, the same group [17] presented a surface-functionalized PDMS microchannel assembled with an interdigitated array (IDA) gold electrode for the detection of human cardiac troponin I (cTnI) in the diagnosis of acute myocardial infarction (**Fig. 3**). In this case, and in order to eliminate electrode fouling, the surface functionalization of the PDMS channel was performed previously to assembling the PDMS channel with an IDA chip. Silanization process was performed onto the internal surface of the PDMS channel, excluding the gold electrodes, with surface-exposed carboxyl group. Protein G, which specifically binds with the Fc portion of the IgG antibodies, was immobilised on the silane film and followed by anti-cTnI, enhancing proper orientation of the antibody and free movement of Fab arms while forming tertiary structures without interfering with the antibody´s ability to bind the antigen. Following a sandwich assay format with AP-labelled secondary antibody, electrochemical signal of the enzymatic product is detected at the IDA microelectrode by cyclic voltammetry. A low detection limit of 148 pg mL^{-1} cTnI within 8 min was obtained, which is attributed to the antibody´s proper orientation by protein G and the best packing density reducing steric hindrance and no fouling on the gold electrodes via the surface-functionalized PDMS channels.

In the article of Liu et al. [18] (ACA2009), a PMMA microchannel was modified with poly(ethyleneimine) (PEI), an amine-functional polymer capable of being coated onto the PMMA surface to introduce amine groups, which can be widely used to immobilise the specific antibody. With the aid of glutaraldehyde as a linker, covalently immobilisation of anti α-fetoprotein (AFP) hepatocellular carcinoma biomarker is performed. Following a sandwich type ELISA, and after injection of the analyte, a HRP-conjugated secondary antibody is employed to determine the concentration of AFP. After addition of H_2O_2 and 2-amino hydroxybenzene, the enzyme HRP catalyse the substrate reaction and the generated electroactive product can be detected by differential pulse voltammetry (DPV). The NH2-modified inner walls of the microchannel can enhance the capacity of the antibody binding on the PMMA surface while keeping its bioactivity and permitting the detection of AFP down to 1pg mL^{-1}.

Another possibility of performing immobilisation of antibodies on the microchannel surface of PMMA has been reported by Wang et al. (Electrochem commu 2008). The authors used PMMA microchannels filled with an Al_2O_3 sol-gel network to encapsulate capture antibodies for IgG as a model analyte. The inner surface of the PMMA microchannel was first treated with a copolymer to bear silanol groups, followed by introducing a solution containing anti-IgG and Al_2O_3 sol-gel to allow polycondensation between the sol-gel and the silanol groups. Therefore, the specific antibody was encapsulated in the alumina gel matrix for the following immunoassay. After binding of the analyte, and injection of ALP-antibody conjugate, electrochemical (DPV) detection by the addition of the substrate p-APP was accomplished. A detection limit of 1 pg mL^{-1} showed the high sensitivity and viability of this immobilisation procedure which preserve the bioactivity of antibodies and resist non-specific adsorption.

4.1.2 Antibody immobilisation onto microbeads

The use of microbeads as immobilization support entails some advantages such as the surface to volume ratio is highly increased even in comparison with microchannel immobilization. When the beads are dispersed, diffusion distances are reduced and the higher efficiency of interactions between samples and reagents rend better sensitivity. Furthermore, the antibodies or antigens attached onto the beads can be easily transported in a fluidic system. A highly control of the antibody load into the channel and fast replacement of the beads between assays are added benefits. By other hand, the high variability of surface modification available on these microbeads introduces multiple functionalities to a single microfluidic design. Also, the possibility of incorporating microbeads into microfluidic assays allows many different strategies to immobilize the beads in the channels for performance reactions and detection. However, microbeads have the risk of adsorbing to channels and electrodes, clogging channels, increasing the flow resistance and scattering the light.

Microbeads can be either non-magnetic or magnetic and different antibody immobilisation methods can be implemented.

a) Non-magnetic microbeads

The most common materials are polystyrene or glass due to its known properties and surface chemical modifications. In both cases, a physical retention microstructure is necessary to facilitate the removal of unbound analyte or antibodies.

Ko et al. [20] developed a system for simultaneous determination of three cancer biomarkers (AFP, CEA, and PSA). Based on a sandwich format, polystyrene beads were used to immobilize the specific antibodies through the avidin-biotin system. The modified beads were then trapped in the reaction chamber by PDMS pillar-type microfilters. After capture of the sample-containing biomarkers, gold nanoparticles-labelled secondary antibody together with a silver enhancer is used for detection. Gold nanoparticles catalyse the reduction of ions silver into metallic silver, which after deposition increase the size of the gold nanoparticles. As a result, the microbeads are connected to each other and form an electrical bridge between a set of underlayered platinum microelectrodes reducing the resistance (**Fig. 4**). The same group reported two similar microfluidic immunoassays where only AFP or anti-protein A antibody were determined using a single channel microdevice [21,22]. Dramatic reduction in the analysis time and sample volume in comparison with conventional ELISA was obtained, while a simple, high sensitivity and real time monitoring was made by measuring the electrical resistance.

Yoo et al. [23] have reported a single channel microfluidic device for hippuric acid (HA) determination (**Fig. 5**). In this case, a dam traps polystirene beads, where the specific antibody is immobilized. Following the interaction between antigens (HA and Ferrocene derivatized HA) and immobilized antibody, the eluted Fc-HA passes across the dam to the detection channel. Onto a gold electrode and amplified by using the enzyme GOX, the current associated with the unreacted Fc-HA was measured by cyclic voltammetry. Reduction in the assay time (1 min compared with 20 min for conventional electrochemical HA immunoassay system) and sample volume were some of the benefits of the developed system.

By other hand, controlled-pore glass microbeads (CPG) have also been used for heterogeneous microfluidic immunoassays. Aminopropyl modified CPG reacted with glutaraldehyde as a linker can be used for immobilization of the immunoreagents. Besides the chemical modification, the group of Raba et al. has reported different trapping mechanisms for the glass beads and application to the analysis of various

analytes. In the work of Panini et al. [24] CPG modified with the specific antibody against PSA were affixed on a disk surface by spread on one side of a double-coated tape. This disk can rotates to minimize diffusional constrains as part of a continuous/stopped flow operation microsystem. Following a sandwich format, a HRP enzyme-labelled second antibody in presence of H2O2 catalyses the oxidation of 4-tert-butylcatechol, whose back electrochemical reduction was detected on a carbon nanotube-modified glassy carbon electrode. The system allows quantification of PSA in human serum samples diminishing the overall assay time (30 min) and showing higher sensitivity respect to ELISA commercial test kits. Following the same principle for antibody immobilization on CPG, rotating disk and operational procedure, Arevalo et al. [25] have determined progesterone in bovine serum samples. Besides and within the same group, Messina et al. [26] developed an analogous procedure for determination of an inflammatory cytokine (IL-6). After immobilisation of the specific antibody onto the aminopropyl-glutaraldehyde-modified CPG, these microbeads were blocked into the central channel of the microdevice by means of glass fibres. Then, sample and reagents were injected sequentially through the beads bed in a sandwich format. After immunological interaction, the product of streptavidin-alkaline phosphatase conjugate was measured on the surface of a gold electrode at the end of the central channel. In another example and using a similar and simple plexiglas design, Pereira et al. [27] used a diameter decrease of the central channel to prevent the modified CPG escape. IgG anti-gliadin human antibodies related to celiac disease were electrochemically determined in a fast and sensitive way for diagnostic information.

b) Magnetic microbeads

Magnetic beads are usually made with an iron core (so they are paramagnetic) and covered with polystyrene for chemical surface modifications. Their main advantage comes from its easy manipulation by magnetic fields. This fact eliminates the need for physical retention microstructures allowing the beads to be immobilized and released at different stages and desired areas of the assay. Although the beads can be immobilized on the electrode surface (electrochemical sensors), they are potentially advantageous when they are dispersed or resuspended in large sample volumes for the capture step reducing the diffusion distances between analyte and antibody. After being collected by a magnet, the remaining assay steps may be performed under favourable microfluidic

conditions. By other hand, the beads are commonly available with reactive functional groups in order to facilitate the attachment of antibodies or other molecules.

Magnetic beads modified with amino groups and with the aid of glutaraldehyde as a linker have been used to immobilize antigen or specific antibody in microfluidic immunoassays. Fernández-Baldo et al. [28] and Pereira et al. [29] used this methodology to immobilize the antigen for determination of *Botrytis cinerea* in tissue of fruits and human serum IgG antibodies to *Helicobacter pylori* respectively. In the first case, *B. cinerea*-modified beads were mixed with the sample and specific antibody for competitive immunological reaction. Subsequently, beads were recovered by an external magnet and injected in the microfluidic device where they were located at a specific area of the channel by the aid of a permanent magnet. After the injection of a second antibody modified with the enzyme HRP, electrochemical determination can be accomplished on the surface of a SPCE. A low detection limit of 0.008 μg mL^{-1}, CV lower than 7% and a total assay time of 40 min were obtained. By other hand, *H. pylori*-modified beads were used to retain the specific IgG present in serum samples injected across the channel where beads were located. The signal produced by an alkaline phosphatase enzyme-labelled second antibody specific to human IgG was determined on a gold electrode situated in the central channel of the device. Reduction in the detection limit with respect to conventional ELISA from 2.1 U mL^{-1} to 0.37 U mL^{-1}, CV lower than 5% and total assay time of 25 were obtained following the electrochemical microfluidic immunoassay.

Following a similar procedure for attaching the antibody to the beads, Martinez et al. [30] and Panini et al. [31] determined ethinylestradiol in river waters and zearalenone in feedstuffs samples. In both cases, the resuspended specific antibody-modified beads are allowed to react with the samples outside of the microfluidic device before its injection and retention into the channel for subsequent assay steps. Fast and sensitive assays were reported under these conditions.

Do et al. [32] developed a lab-on-a-chip for magnetic bead-based immunoassay consisting on a magnetic bead-based separator, an interdigitated array microelectrode, and a microfluidic system, which are fully incorporated into a cyclic olefin copolymer substrate (**Fig 6A**). In this work, magnetic beads are commercially prepared with a class antibody (rabbit anti-mouse IgG) and retained in a separator with electroplated

permalloy microarray on a polymer substrate with external permanent magnets. The separator was designed to have a 100 x 50 array electroplated permalloy pattern, and each permalloy pattern has 20 μm in width, 20 μm in length and 1 μm in thickness, fabricated by conventional lithography techniques and electroplating (**Fig. 6B**). This microarray separator permitted a good distribution and strict control of the amount of retained beads for determination of mouse IgG as a model analyte in a total integrated device.

In another example, Bunyakul et al. [33] used superparamagnetic streptavidin-modified beads for immobilization of biotinylated-anti cholera toxin subunit B antibodies. In this work, a mixture of modified beads, the toxin in the sample and a ganglioside GM_1 specific receptor bound to liposomes encapsulating a marker were injected into the microfluidic device and retained by an external magnet. For liposome-based signal amplification, a detergent solution is injected through an additional channel in order to lyse the liposomes and release the marker. Comparison between fluorescence and electrochemical detection is accomplished using a dye (sulforhodamine B) and electroactive compound (hexocyanoferrate II/III) as encapsulated markers respectively. Lower detection limit together with a higher flexibility and reliability of signal recording was found for the electrochemical detection showing its very suitable characteristics.

A selective orientation of the specific antibodies can be accomplished using protein A/G as biological linkers bound to the magnetic beads. These bioaffinity proteins selectively immobilizes the Fc regions of the antibody enabling favourable orientation which results in higher antigen capture efficiency, minimized steric hindrance and avoid protein denaturation. Recently, our group have propose a lab-on-a-chip strategy integrating all analytical steps of the immunoassay protocol for zearalenone myoctoxin detection, where the specific antibody was immobilized onto proteinG-mofidied-beads [7]. Furthermore, in this work we have designed a strategy that implies the creative definition and use of the simple design of double-T microchip geometry in order to perform the complete electrochemical immunoassay. The strategy was based on the use of the two channels of its layout, injector and separation channels, to perform the immunocompetition and the enzymatic reactions, respectively. The complete analytical strategy is shown in **Fig 7.** The injector arm has been termed the immunological

reaction chamber (IRC), and it is the microchannel where the immunological complex formation between antibody-coated beads and sample or enzyme-conjugate takes places (**Fig. 2A**). For the second stage, the modified beads were dragged to a region of the longitudinal channel near the detector, called the enzymatic reaction chamber (ERC) by the aid of a removable magnet. In this area, the enzymatic product is obtained from the antibody-retained HRP-conjugate and then detected (**Fig. 2F**). This approach is based on physical separation of both zones avoiding, in an elegant way, one of the greatest problems associated with microfluidic immunoassays as nonspecific adsorption to the channel walls surfaces. The use of magnetic beads that can be easily handled and double-T geometry of the chip was especially appropriate for this configuration. After optimisation of the different stages in both areas of the microfluidic, the immunoassay was performed for the analysis of zearalenone showing a suitable limit of detection (0.4 $\mu g\ L^{-1}$) and extremely low systematic error of 2% from the analysis of a certified reference material. In the same way its reliability was demonstrated by the analysis of selected infant foods with recoveries of 103 and 101% for solid and liquid samples respectively.

4.1.3 Antibody immobilization onto the electrode surfaces positioned within the microchannels

Another possibility to immobilize the antibodies within the microfluidic channels implies their direct attach onto electrode surfaces usually located as a wall of the channel. This option has some of the advantages and drawbacks already commented for immobilization on microchannel surface together with those derived from the intimate contact between the biological receptor and the electrochemical transducer (immunosensors). Different substrates can be used as electrode material. However, carbon derivatives, platinum, and mainly gold are the most used in microfluidic devices. Different geometries are also used, where interdigitated array electrodes (IDA) are ideally suited for using with microfluidic due to the additional sensitivity provided. The IDA consists of an electrode pair in which the microelectrode “fingers” of the two electrodes are interdigitated. The IDA provides additional sensitivity when the two interdigitated electrodes are held at different potentials to cause redox cycling of the electroactive species to be detected. By other hand, a variety of surface chemistries can

be employed for antibody immobilization. In this sense, the well-known quimioadsorption on gold electrodes is predominant.

Zou et al. [34] presented a nano IDA integrated into a microfluidic COC polymer substrate. In this case, the authors used impedimetric detection for measuring the immobilized amount of mouse anti-rabbit IgG as a proof of concept. The immobilization of protein is based on the formation of self-assembled monolayers (SAMs) of alkanethiols groups on gold electrode surfaces. The surface was activated by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDAC) and n-hydroxysuccinimide (NHS) which can be covalently bound to mouse anti-rabbit IgG owing to its capacity to recognize amino groups of proteins. Different sample solutions with different concentrations of the protein were injected into the sensing microchannel and held for incubation during 10 min. Based on the concentrations of the biomolecule bound to the surface, its yield different changes in electrical impedance between the electrodes of the IDA. However, at this point no selective analyte recognition was performed in the device.

The group of O´Sullivan [35] produced a microfluidic cell for the multiplexed electrochemical detection of cancer markers. The electrochemical cell consisted of 16 gold working electrodes arranged in a four by four electrode array on a chip measuring 21 mm x 23 mm, where each working electrode was placed between one silver pseudo-reference and one gold counter electrode. Antibodies were immobilized on the electrode array via the self-assembled monolayer strategy. Each electrode was modified with a SAM of a dithiolated derivative of benzoic acid baring a PEG moiety and COOH group. After activation with EDC and NHS, the specific antibodies were incubated and residual groups were blocked with ethanolamine hydrochloride. Thus provide hydrophilic terminal groups, which in combination with the PEG moiety suppressed non-specific binding. Detection was carried out either impedimetrically by measuring the change in charge transfer resistance of the system before and after addition of the target antigen; or by a sandwich immmuno-complex between the target and an anti-biomarker/HRP conjugate using differential pulse voltammetry (DPV). Carcinoembryonic antigen and prostate-specific antigen were simultaneously measured in alternative electrodes with high reproducibility (<8%).

Using a microfluidic chamber, Sun et al [36] developed an immunoelectrochemical sensor for determination of salivary cortisol concentration (**Fig. 8**). Microfabricated IDA gold electrodes encased in a microfluidic chamber were functionalized, by means of thioctic acid, EDC and NHS-biotin solutions, to immobilize the cortisol capture antibodies. Following a sandwich format, the enzymatic product generated by alkaline phosphatase attached to detector antibodies was measured by cyclic voltammetry. The method accurately measured cortisol in collected saliva samples achievec to a concentration of 0.76 nmol/L with an incubation time of 10 min.

In the work of Fakunle and Fritsch [14] the microchannels were constructed from LTCC materials with screen-printed gold electrodes situated on the four walls. Two of the SPG electrodes span the top and bottom walls and serve as the auxiliary electrode and the assay site respectively. The other two are centred lengthwise on the sidewalls of the channel. One serves as the working and the other as the pseudoreference electrode. The primary antibody (rat anti-mouse IgG) was immobilized on a SAM of 11-mercaptoundecanol (MUOL) activated with EDC onto the bottom SPG electrodes. After successive addition of the sample (mouse IgG) and a secondary antibody AP conjugated following a sandwich approach, the enzymatic product was monitored by CV, using a total analysis time of 60 min.

In another example Pereira et al. [37] described a microfluidic immunosensor for the detection of IgG antibodies specific to *Echinococcus granulosus* in human serum samples for the immunodiagnosis of hydatidosis. In this work, a gold electrode situated at the central channel of a plexiglass platform was modified with Au nanoparticles deposited on SAMs of 1,6-hexanedithiol onto the gold electrode surface. The Au nanoparticles were put in contact with a 3-mercaptopropionic acid solution. Here, the –SH group of mercaptopropionic acid reacted with the surfaces of particles leaving as a result, free COOH groups for immobilization of the antigen previous activation with EDC and NHS solutions. The immunoassay was conducted by allowing the IgG-specific antibodies to *E. granulosus* present in the samples reacted with the antigen immobilized on the top of the Au nanoparticles. Quantification was made using HRP-labelled sencondary antibodies specific to human IgG and amperometrical detection of the enzymatic product on the gold surface electrode. Due to the increase of the active area through the use of gold nanoparticles an improvement of sensitivity and stable immobilization of biomolecules was assessed. In this sense, a limit of detection of 0.091

ng mL^{-1} and a coefficient of variation lower than 4.1% were obtained with this microfluidic immunosensor.

Other possibility to immobilize biomolecules on the gold electrode surfaces within microchannels is through the use of monolayer of poly dendrimers. In the work or Park et al. [9] the antigen 2,4-dinitrophenylacetic acid (DNP) modified surface is constructed onto a gold electrode for microfluidic immunosensing of anti DNP antibodies. In a first step, an amine-reactive SAM is produced where a poly(amidoamine) G4 dendrimer is attached. Subsequently, a succinimidyl ester of DNP is allowed to react. N-Hydroxysuccinimide (NHS) groups, which located at the end of activated-DNP, react with the plenty of amine groups on the dendrimer for its immobilization. As a proof of concept of this microfluidic device, where the transport of multiple liquids without any external equipment is remarkable (see next section), GOX tagged anti-DNP antibodies were used as analyte. After loading the sample into the reaction channel and interaction with the antigen-functionalized electrodes, the electrochemical signal was produced by the GOX enzyme using ferrocennemethanol as the electron mediator and glucose as the substrate. In a later work of the same group [10], they follow the same immobilization procedure to form the dendrimer-DNP surface. However, in order to avoid the pre-treatment of the sample tagging the antibodies with the GOX enzyme, a backfilling method was used. That means the next steps: (i) The non-labelled native antibodies, which would be substituted by real sample, reacted with the functionalized antigen surface. (ii) The remaining surface amine groups on the dendrimer layer are covalently covered with periodate-treated GOX which is amine reactive. (iii) Now, from the bioelectrocatalytic reaction with the immobilized GOX and the electrochemical signal registration, the surface density of biospecifically bound antibodies could be quantified.

Other electrode materials have also been used for antibodies immobilization within the microchannels. Nashida et al. [14] presented a microfluidic device consisted on four flow channels extended from four solution reservoirs to a reaction chamber formed at the center. In the reaction chamber, a platinum-working electrode (400 μm diameter) was formed for antibody/antigen immobilization and electrochemical detection. The antigen or specific antibody (human α-fetoprotein –AFP-) were immobilized on the platinum working electrode by embedding in a double layer of plasma-polymerized film (PPF) using hexamethyldisiloxane as monomer. In both cases, the interaction was

characterized by using a GOX-labelled anti-AFP antibody. The enzyme catalysed the oxidation of glucose, and gluconolactane and hydrogen peroxide were produced. The hydrogen peroxide was electrochemically oxidized on the platinum working electrode and the generation of current detected. The system although in a previous state of development has the potential for biochemical analysis.

Carbon screen-printed electrodes modified with electropolymerized polypyrrole propylic acid (PPA) as immobilization matrix have been used for immunosensing of mouse IgG as a model analyte into a potential multiplexing microfluidic system [38]. This PPA surface was activated with EDC and NHS for covalent immobilization of the specific antibody goat anti-mouse IgG. Using a sandwich format and AP as conjugated enzyme, the electrochemical product was measured by cyclic voltammetry. This microfluidic device was fabricated by screen printing technology, involving inexpensive and simpler conditions.

4.2 *Homogeneous microfluidic immunoassays*

In the case of homogeneous immunoassays, the formation of the antibody-antigen complex must produce a change in the label´s signal. No separation step is required to distinguish between the bound and unbound states of the immunospecies. This fact present some advantages over heterogeneous configuration since there is no need for antibody immobilization and rinsing steps, and there is no solid phase that can be affected by non-specific adsorption. However, homogeneous assays usually suffer of poorer limits of detection and selectivity than heterogeneous assays [1]. That made homogeneous assays being used for analytes with relative high concentrations as therapeutic drugs in blood, and they are particularly effective in partially purified samples (environmental water samples and food) containing interferences that reduce the sensitivity of the assay. The most popular form of homogeneous immunoassay is based on capillary electrophoresis (CE), in which the immune complex and free antibodies are discriminated based on their electrophoretic mobilities. CE-based microfluidic chips have become very popular because of their compatibility with electrokinetic fluid manipulation fast electrophoretic separation, combined with great capability for integrated sample preparation and enormous potential for multiplexing [39]. Although most microfluidic immunoassays have used LIF for detection, some electrochemical examples are presented in the literature. Recently a MCE enzyme

immunoassay for carcinoembryonic antigen and alpha-fetoprotein using electrochemical detection has been reported by Zhang et al. [11]. The CEA and AFP were incubated simultaneously with two relating enzyme-labeled antibodies in the sample tube. Then, the immunocomplexes were injected into the sample reservoir and separated. After addition from another reservoir of the enzymatic substrate, the product was detected amperometrically at the end channel electrochemical cell. Following a non-competitive format the four immunospecies (Anti-AFP-HRP, anti-CEA-HRP, AFP-anti-AFP-HRP, and CEA-anti-CEA-HRP) are separated and detected in less than 60 s.

5. Multiplexing designs

The use of a single microfluidic device for the analysis of multiple samples or analytes is gaining importance as a new direction in the field of lab-on-a-chip or micro total analysis system. This aspect is especially important in the field of biological or clinical diagnosis where samples contain more than one analyte, or multiple samples have to be analysed.

Beneath this principle, different strategies are reported in the literature. However, in the case of electrochemical microfluidic immunoassays, they usually exploit the use of parallel microchannels immunoassays or arrayed microelectrodes. Different multiplexing examples are described below, while the other aspects have been already commented in the corresponding section.

In the case of Dong et al [38], they used thick-film screen-printing technology to fabricate a multiplexing microdevice using a polyester board where electrodes and wires are printed, microchannels moulded and finally sealed with PDMS. In the reported configuration, an inlet mainstream channel distributes the solutions into eight parallel manifold microchannels where the array electrodes are located, and then converges to the outlet mainstream channel (**Fig. 9**)). As already mentioned in the previous section, specific antibodies are immobilised onto PPA modified electrodes to perform a sandwich format immunoassay, using mouse IgG as a model analyte. Although the device is demonstrated with one-analyte detection, multi-analyte sensing can be realized after simple modification of the design.

Henry et al. [35] developed a low-density electrochemical array for the detection of tumour markers. In this work, 16 gold working electrodes are arranged in a four by four electrode array on a microchip where a sandwich format immunoassay was accomplished. In the prototype presented, the electrode array was incorporated into the microfluidic device where fluidic channels, reservoirs, valves and connections are integrated into an automated delivery system (**Fig. 10**). Used in a single-channel format, up to 16 electrodes can be exposed and addressed at the same time for determination of CEA and PSA.

In another example of multiplexed electrochemical immunoassay, Ko et al [20] fabricated the microchip shown in **Fig. 11**. The system is composed of a glass layer where electrodes are formed for detecting electrical signals resulting from immunoreactions, and a PDMS layer where microchannels, inlets and outlet are designed. They layout comprised a main inlet for samples and reagents, an inlet for the 1st antibody immobilized microbeads for each antigen, and an outlet. Furthermore, the main inlet is connected to a wide microchannel (600 μm) subsequently divided two by two, which finally are connected with four reaction chambers (about 7 μL). In the middle of each reaction chamber and following a sandwich format, the immunocomplex-microbeads are retained and place them onto the electrode by the use of micropillars for detecting the immunoreactions. This interesting approach is demonstrated by simultaneous determination of CEA, PSA and AFP biomarkers.

By other hand, the group Reymond [15] reported a robotized microfluidic platform for automated immunoassay, previously described in the fluid delivery section. The immunochip is comprised of a linear array of eight independent microchannels with gold electrodes incorporated. As in conventional 96-well microtiter plates, the microchannels have a standard spacing of 9 mm to facilitate automation being fully compatible with conventional pipetting devices. Each immunochip is capable of running 8 assays in parallel (**Fig. 12**). The hole system, which includes a robotic liquid-handling station, allowed performing up to 4 x 96 tests unattended and yields 16 results every 9 min, postulating as a suitable example of automated microfluidic immunosensing system. A similar eight channels device was previously reported by the same group [40] for determination of folic acid as application example.

Another possibility for multiplexing microfluidic immunoassay relay on the use of MCE. Zhang et al. [11] reported an MCE electrochemical enzyme immunoassay protocol for the determination of CEA and AFP. Free-labelled antibody and Ag-Ab* complex produced in solution can be electrophoretically separated into the microchannel and after a postcolumn reaction and then traced by the enzyme substrate reaction, were amperometrically detected.

6. Conclusions and future aspects

The aim of this review has been a brief description of the current state of the art of electrochemical microfluidic immunoassays. The combination of the properties derived from immunoassay and microfluidic platforms has proven to be a valuable tool on the miniaturization and simplification tendencies of analytical science for μTAS or lab-on-a-chip scene. Different areas from these systems have been studied as materials substrates, fluid handling, multiplexing approaches and special attention was focused on the different antibody immobilization strategies. In this sense, pros and cons of using microchannel-wall-surfaces, microbeads or electrode surfaces within microchannels, as immobilization support have been discussed. By other hand, the inherent features of electrochemical detection have made this technique widely expanded for microfluidic systems. Although other interesting detection approaches can be observed in the literature, ED has grown extensively in the last years demonstrating its suitability availed by the number of articles reported.

The potential progress in microfluidics immunoassays is enormous due to its excellent properties for the analysis of concern samples, mainly in the biological and clinical or medical fields, although other subjects such food analysis and environmental are also important. However, this technology involves the knowledge from different areas of science and engineering, which must grow in parallel to get the final goal. In the case of materials substrates, although polymers are still the dominant material in this area, important limitations already exist in practical applications. This fact demands the need for massive improvement in the surface modification and immobilization methodologies. This is a challenging issue in microfluidic immunoassays, not only because is a key factor for preventing non-specific adsorption but also it may greatly

impair the sensitivity and selectivity of the immunoassay. Besides, fluid handling although usually carried out trough electrokinetic or pressure-driven flow needs from the advancement in areas such as passive actuation of pumps, valves, mixers to rend automatic and facile fluid control, eliminating other macroscopic moving parts. By other hand, multiplexing as the capability for simultaneous multianalyte or multisample analysis probably represents the most exciting development in microfluidic assays. The full integration of all components on disposable and cheap microfluidic systems is the ultimate challenge for commercial applications. A great effort must be conducted in the way to reduce complexity, making systems more robust, user-friendly and suitable for point-of-care diagnostic devices. We think the near future in this area is very promising.

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Table 1. Representative examples of electrochemical microfluidic immunoassay systems reported in the literature from 2005.

Analytes	Substrate	Immunoassay format	Antibody/antigen Immobilization	Fluid delivery	Detection	Multiplexing	Ref.
Progesterone	Plexiglas	Heterogeneous competitive	CPG beads	Syringe pump	Amperometry	No	[25]
Cholera toxin subunit B	Glass-PDMS	Heterogeneous sandwich	Magnetic beads	Syringe pump	Amperometry and coulometry	No	[33]
Mouse IgG	COC	Heterogeneous sandwich	Magnetic beads	Syringe pump	Chronoamperometry	No	[32]
Mouse IgG	Polyester-PDMS	Heterogeneous sandwich	Carbon-SPE modified with PPA	Syringe pump	CV	Si (8 channels)	[38]
Mouse IgG	LTCC	Heterogeneous sandwich	Gold electrode	Capillary and hydrostatic pressure	CV	No	[8]
Botrytis cinerea	Plexiglas	Heterogeneous competitive	Magnetic beads	Syringe pump	Amperometry	No	[28]
CEA, PSA	Polycarbonate	Heterogeneous sandwich	Gold electrode	Syringe pump	Impedimetry and DPV	Si (16 arrayed electrodes)	[35]
Zearalenone	Glass	Heterogeneous competitive	Magnetic beads	Electrokinetic	Amperometry	No	[7]

Analytes	Substrate	Immunoassay format	Antibody/antigen Immobilization	Fluid delivery	Detection	Multiplexing	Ref.
Folic acid	Polyimide	Heterogeneous competitive	Gold electrode	Peristaltic pump	Amperometry	Si (8 channels)	[40]
IgG	Glass-PDMS	Heterogeneous sandwich	PDMS channel wall	Syringe pump	CV	No	[16]
Cardiac troponin I	Glass-PDMS	Heterogeneous sandwich	PDMS channel wall	Syringe pump	CV	No	[17]
CEA, AFP, PSA	Glass-PDMS	Heterogeneous sandwich	Polystyrene beads	Syringe pump	Resistance	Si (4 channels)	[20]
Anti-proteinA IgG	Glass PDMS	Heterogeneous non-competitive	Polystyrene beads	Syringe pump	Resistance	No	[22]
AFP	PMMA	Heterogeneous sandwich	Channel wall	Syringe pump	DPV	No	[18]
AFP	Glass-PDMS	Heterogeneous sandwich	Polystyrene beads	Syringe pump	Resistance	No	[21]
Ethinylstradiol	Plexiglas	Heterogeneous competitive	Magnetic beads	Syringe pump	Amperometry	No	[30]

Analytes	Substrate	Immunoassay format	Antibody/antigen Immobilization	Fluid delivery	Detection	Multiplexing	Ref.
Interleukin-6	Plexiglas	Heterogeneous sandwich	CPG beads	Syringe pump	Amperometry	No	[26]
AFP	Glass-PDMS	Heterogeneous sandwich	Pt electrode	Electrowetting	Amperometry	No	[14]
Zearalenone	Plexiglas	Heterogeneous competitive	Magnetic beads	Syringe pump	Amperometry	No	[31]
PSA	Plexiglas	Heterogeneous sandwich	CPG beads	Syringe pump	Amperometry	No	[24]
Anti-DNP	Glass-PDMS	Heterogeneous competitive	Gold electrode	Hydraulic pressure	CV	No	[9]
Anti-DNP	Glass-PMMA-PDMS	Heterogenous competitive	Gold electrode	Hydraulic pressure	CV	No	[10]
Anti-Echinococcus granulosus	Plexiglas	Heterogeneous competitive	Gold electrode	Syringe pump	Amperometry	No	[37]
Anti-glyadin	Plexiglas	Heterogeneous sandwich	CPG beads	Syringe pump	Amperometry	No	[27]

Analytes	Substrate	Immunoassay format	Antibody/antigen Immobilization	Fluid delivery	Detection	Multiplexing	Ref.
Anti-Helicobacter pylori	Plexiglas	Heterogeneous sandwich	Magnetic beads	Syringe pump	Amperometry	No	[29]
IgG	Plyimide	Heterogeneous sandwich	Magnetic beads	Gravity and capillary forces	Amperometry	Si (8 channels)	[15]
Cortisol	Silicon and SU-8	Hetogeneous sandwich	Gold electrodes	---	CV	No	[36]
IgG	PMMA	Heterogeneous sandwich	Sol-gel matrix	Vacuum pump	DPV	No	[19]
Hipuric acid	Glass-PDMS	Heterogeneous competitiveo	Polystyrene beads	Syringe pump	CV	No	[23]
CEA and AFP	Glass-PDMS	Homogeneous MCE	---	Electrokinetic	Amperometry	Si	[11]
CTN-I, CRP	PDMS	Heterogeneous sandwich	Gold beads	Electrokinetic	Stripping voltammetry	Si	[12]
IgG	COC	Heterogeneous non-competitive	Gold electrode	Syringe pump	Impedimetry	No	[34]

Figure 1. Photograph of the fabricated immunosensing LOC before and after the test. Reference [9] with permission.

Figure 2. (A) Configuration of the three-electrode microchip system assembled with the PDMS cannel. WE: working electrode, CE: counter electrode, RE: reference electrode. (B) Schematic view of on-chip immunoassay within surface-functionalized PDMS channel. From reference [16] with permission.

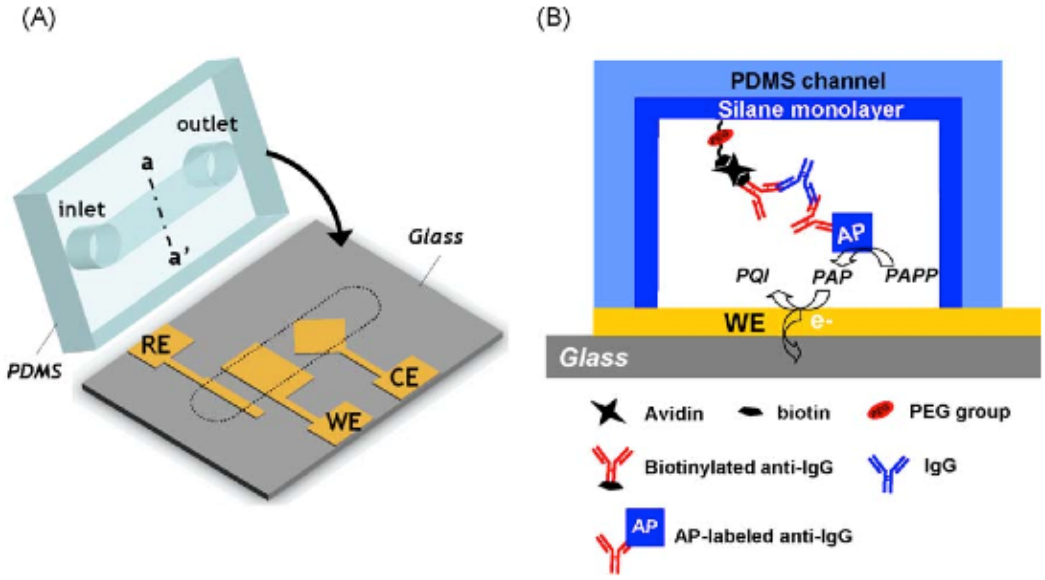


Figure 3. Photograph of the IDA gold chip with three-electrode system and the integrated system. From reference [17] with permission.

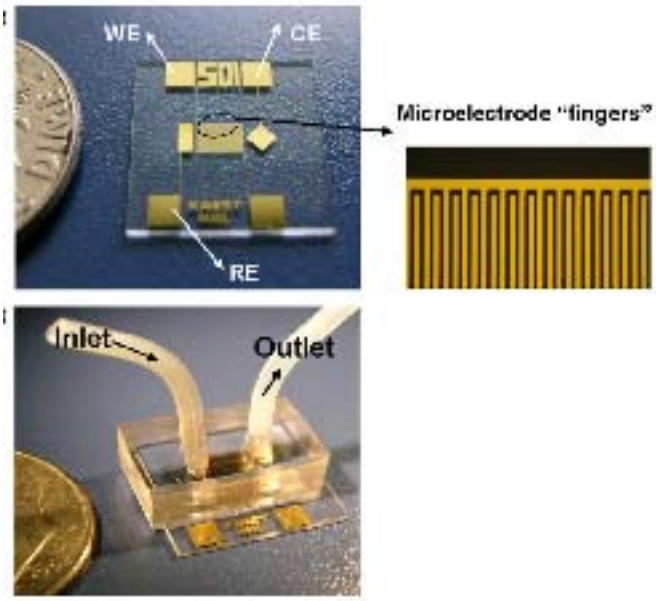


Figure 4. Illustration of electrical detection immunoassay. (a) gathering of the microbeads covered with antigens, (b) antigen-antibody reaction, (c) reaction with gold-conjugated antibody, (d) signal enhancement with silver. From reference [22] with permission.

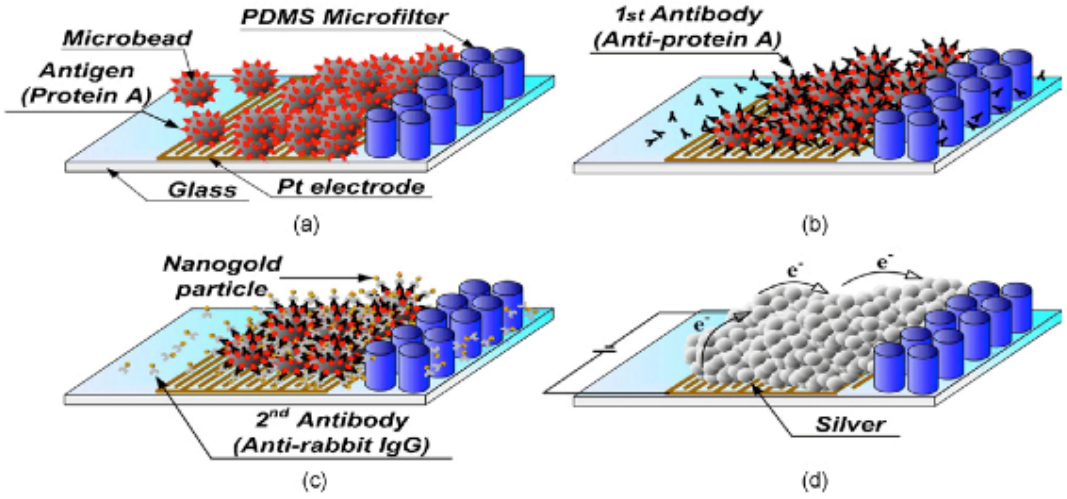


Figure 5. Schematic 3D diagram of the immunoassay chip. (a) The elements of the immunoassay chip and the assembled immunoassay chip. (b) The chamber holding the polybeads [a magnification of a parte of 3 (a)]. Inset: A photograph of the polyebead reserving chamber. (c) A photograph of the immunoassay chip. From reference [23] with permission.

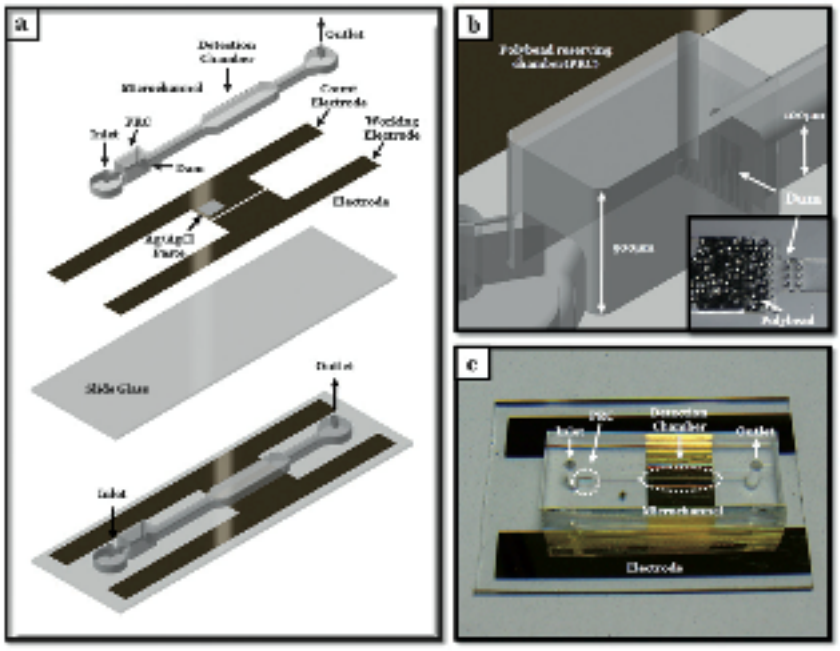
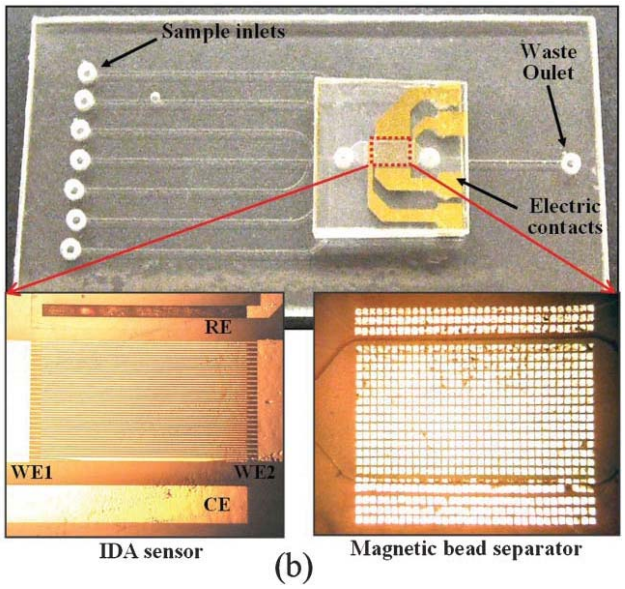


Figure 6. Polymer lab-on-a-chip for magnetic-based immunoassay. (A) Photograph of the integrated device with a magnified view. (B) Magnetic bead separator with electroplated permalloy microarray. (a) Schematic illustration, (b) microphotograph and its SEM picture. From reference [30] with permission.

(A)



(B)

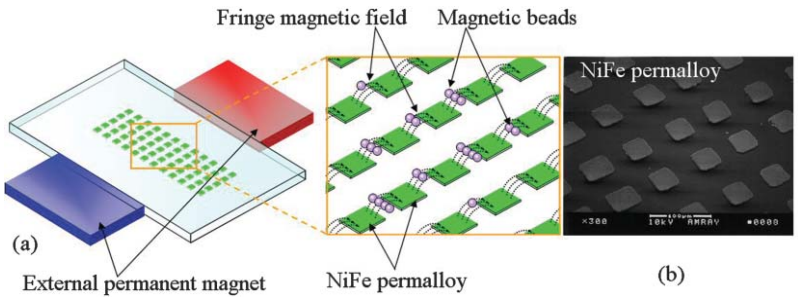
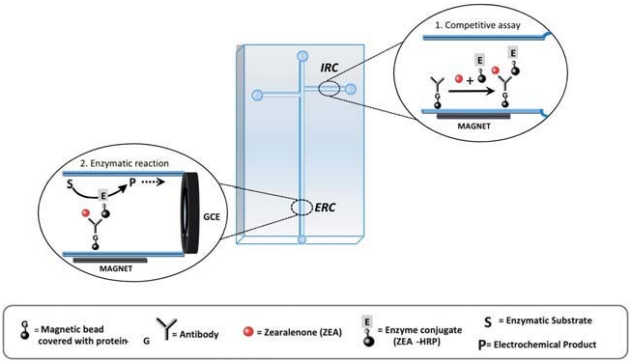


Figure 7. (A) Microfluidic layout and immunoassay principle. (B) Detailed schematic representation of the different analytical and electrokinetic (inset) stages of the electrochemical microfluidic immunosensign strategy. (RB: running buffer reservoir; SR: substrate reservoir; DR: detection reservoir; IRC: immunological reaction chamber; ERC: enzymatic reaction chamber. From reference [7] with permission.

(A)



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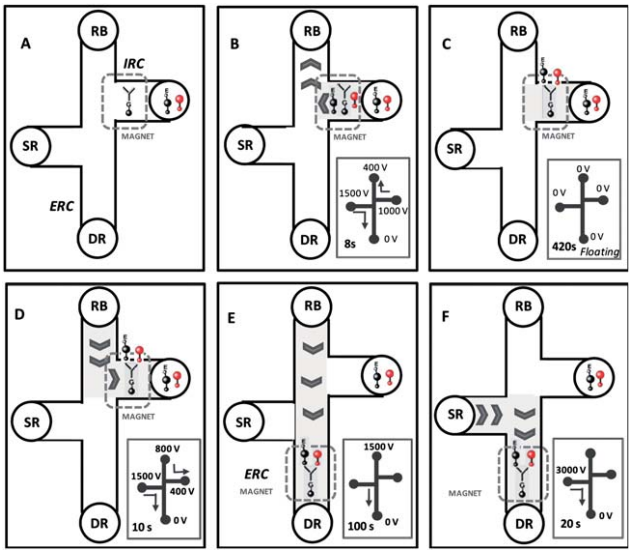
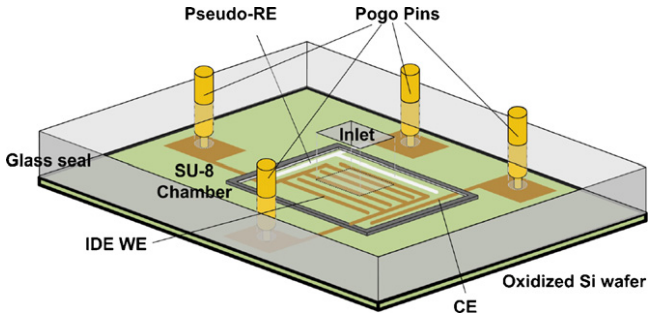


Figure 8. Scheme of the electrochemical cell (A) and sandwich electrochemical immunoassay protocol (B). From reference [36] with permission.

(A)



(B)

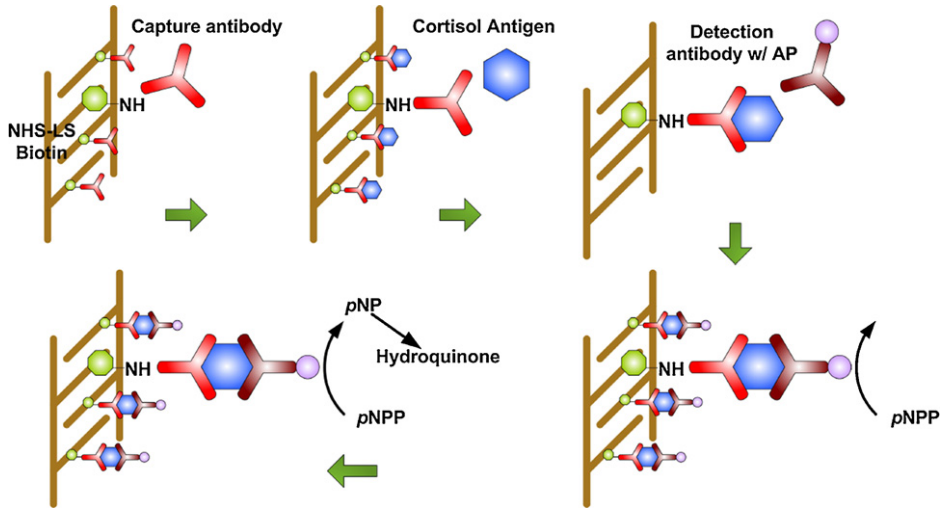


Figure 9. Photos of the multriplexing screen printed microfluidic immunosensor. From reference [38] with permission.

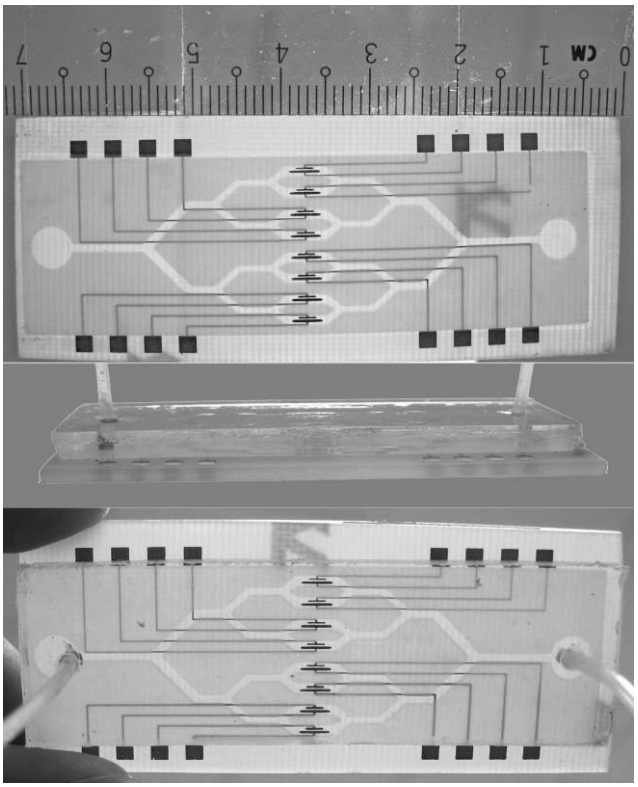
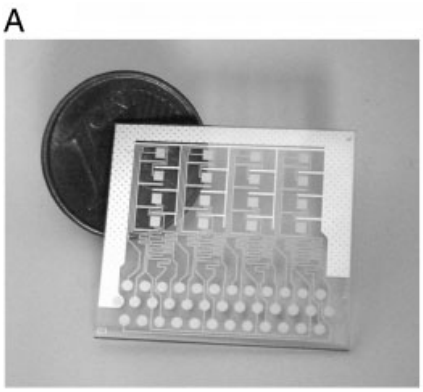


Figure 10. (A) Electrochemical immunosensor array comprising 16 square gold electrodes and common gold counter and silver reference electrode. (B) Atachment of the electrode array to the fluidic chip: (a) support; (b) electrode array; (c) doubleside channelled adhesive tape; (d) fluidic chip; (e) pogo-pin connector; (f) support bar. From reference [35] with permission.



(B)

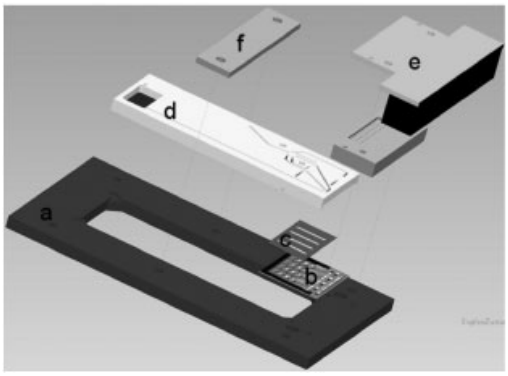
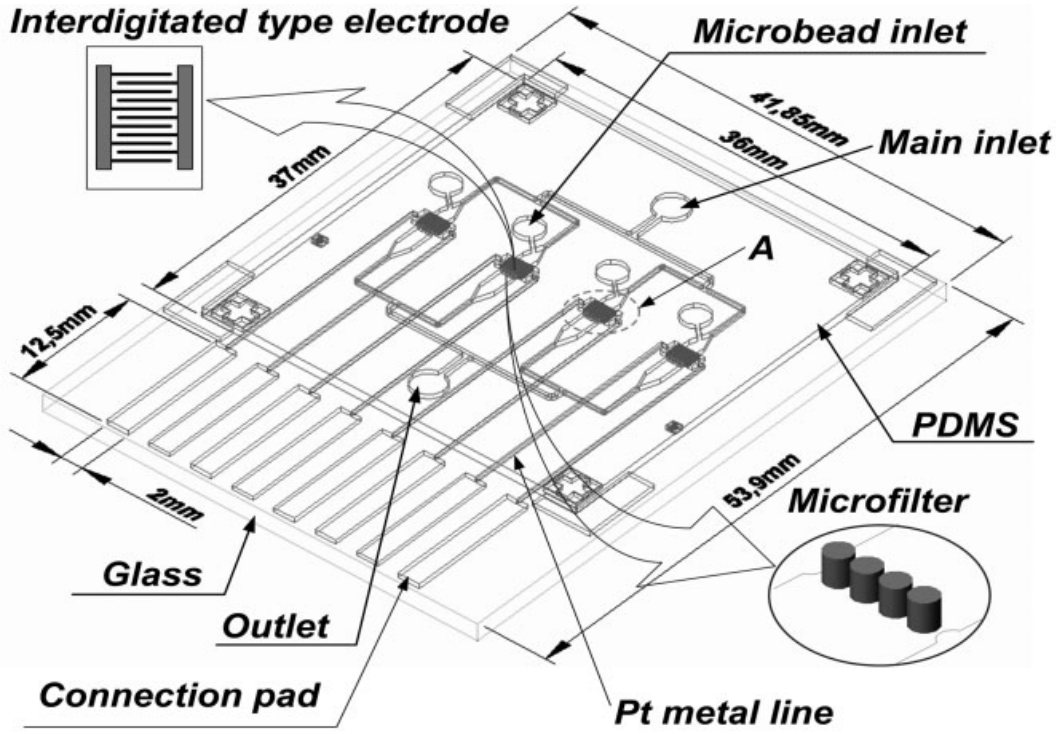


Figure 11. PDMS-glass electro-immunosensing chip. (a) 3-D drawing of design (the detection zone in the chip is indicated as A in the figure); (b) photograph of the fabricated chip. From reference [20] with permission.



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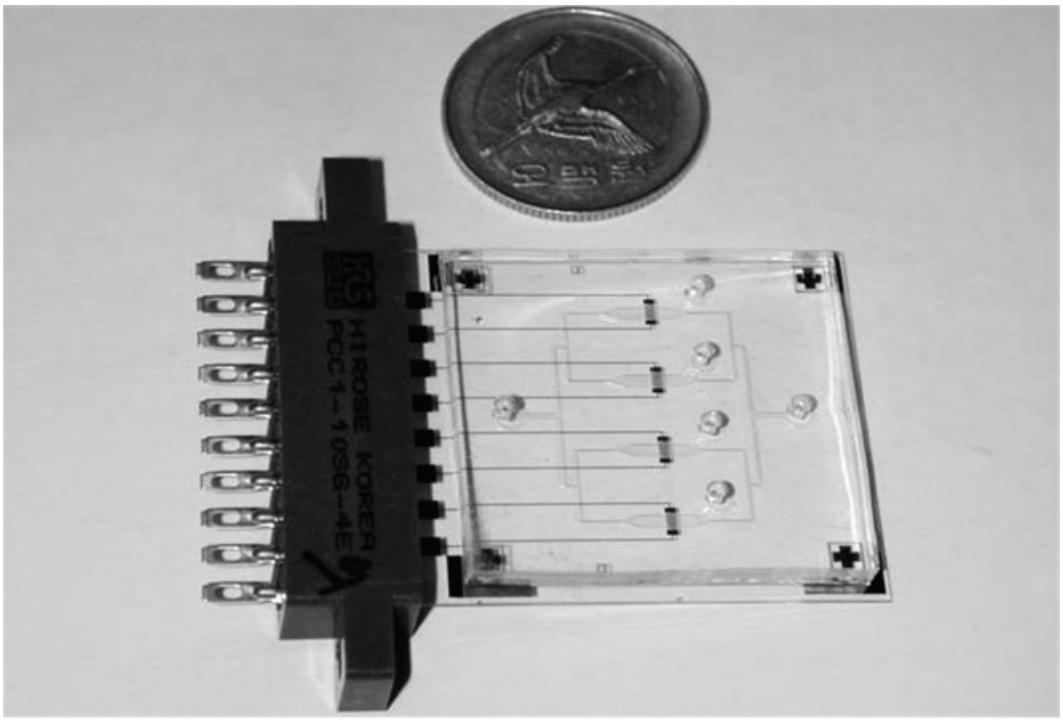
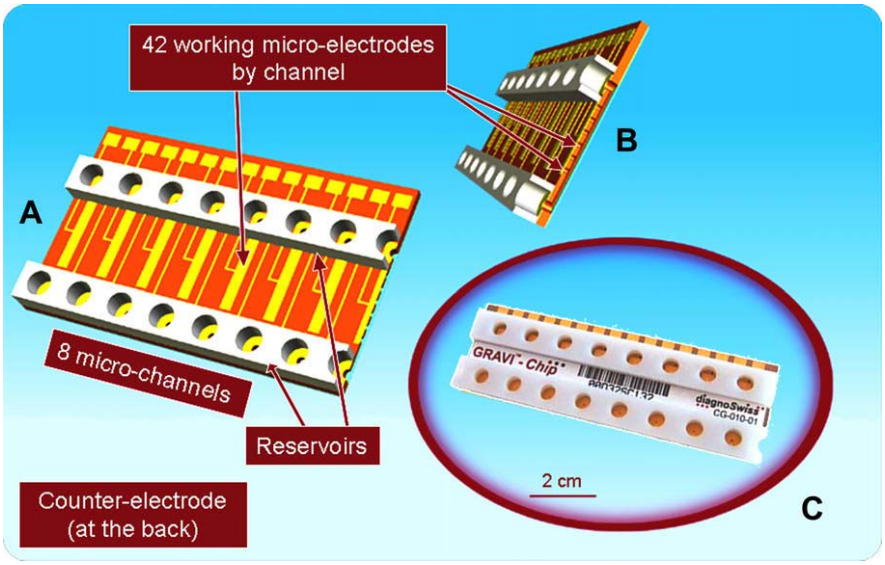


(b)

Figure 12. Vies of the GRAVI-Chip showing (A) upper side of the eight-microchannel chip with its solution reservoirs and electrical network; (B) cross-section of a microchannel with its array of electrodes integrated in recesses; (C) picture of a GRAVI-Chip consumable. From reference [15] with permission.



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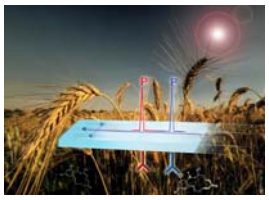

Monitorización electroquímica "*on-chip*" de un método ELISA realizado en placa (*off-chip*) para la determinación de ZEA en alimentos infantiles

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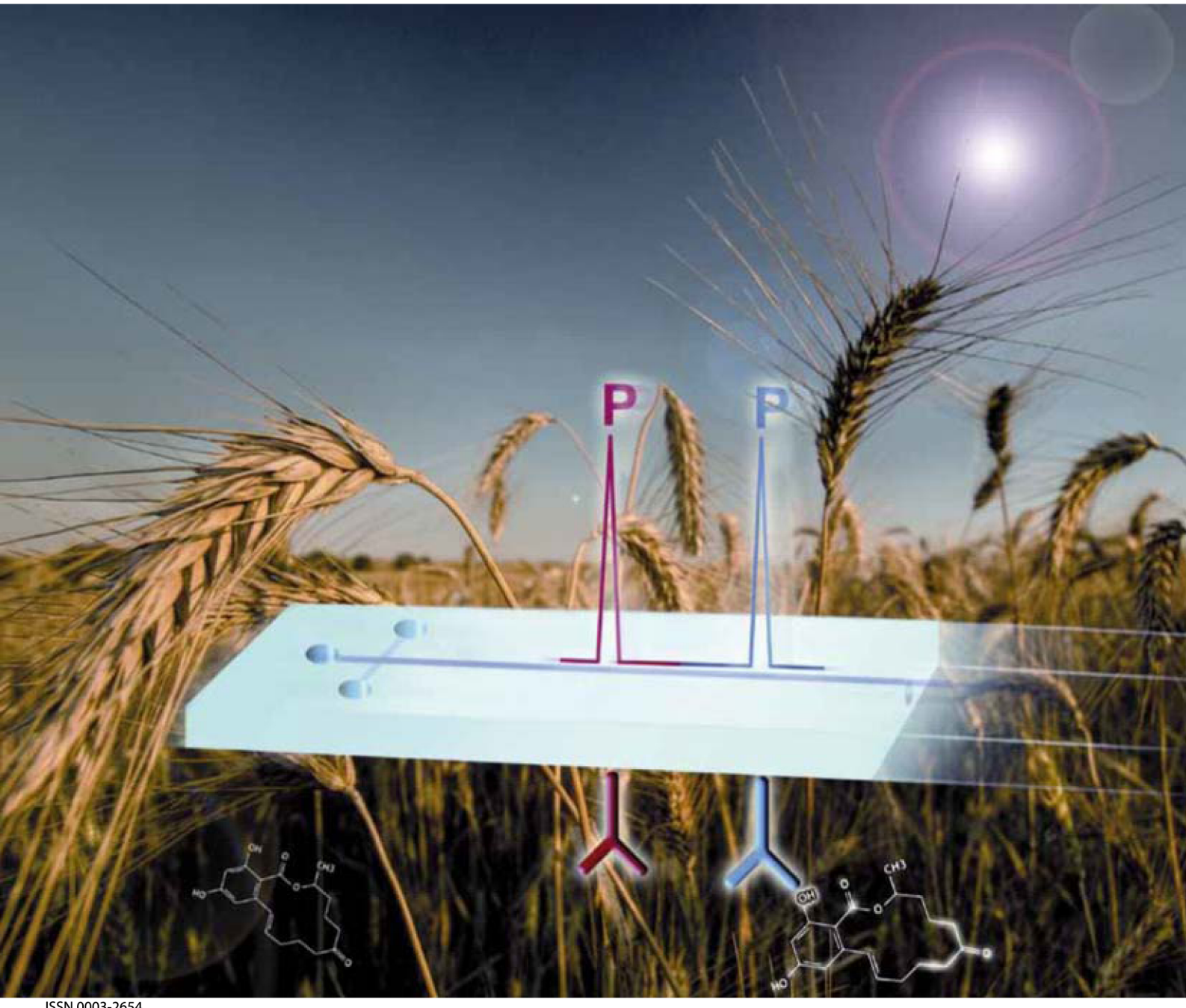

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Electrochemical microfluidic chips coupled to magnetic bead-based ELISA to control allowable levels of zearalenone in baby foods using simplified calibration

Miriam Hervás, Miguel Ángel López and Alberto Escarpa*

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A novel analytical strategy that couples enzyme-linked immunosorbent assay (ELISA) and electrochemical microfluidic chips to determine the mycotoxin zearalenone (ZEA) in baby foods is presented. The analytical cycles for an ultra-fast analysis of the sample and its sequential fast and simplified calibration were performed in about 200 s plus to ELISA protocol. This route avoided the typical four-parameter logistic curve fit which is a highly time-consuming and laborious procedure. An extremely low concentration level of ZEA (less than 1 ppb) was detected with reliability. This level is 20 times lower than the strictest tolerable limit (20 ppb) for baby foods, making the microfluidic approach the newly anticipated analytical security tool for the future. The reliability of the proposal was demonstrated by accuracy evaluations using a certified reference material and by demonstrating its suitability during the control of the regulatory limits of ZEA in baby foods. In addition, the microfluidic approach allowed sensitivity and the incubation enzymatic reaction to be manipulated *in situ*.

Introduction

Microfluidic chips offer faster analysis times, extremely low sample and reagent volumes, as well as the potential for the parallelization of analyses into a small monolithic piece.[1,2] Electrokinetic fluidic motivation is most often used because the flow in multiple channels on a microchip can easily be controlled with only a few electrodes, further simplifying the apparatus by eliminating valves and pumps.[3] Laser-induced fluorescence (LIF) and electrochemical detection (ED) are the routes most commonly used. While LIF, the original detection technique, is the most common detection scheme because of its inherent sensitivity,[4] ED is a valuable alternative due to its inherent miniaturization, without the loss of performance, and because it is highly compatible with microfabrication techniques.[5–7]

Among other analytical uses, microfluidic chips are an ideal platform for performing microscale flow injection analyses.[8,9] This concept includes electrokinetic injection, pumping, and analyte detection using the microchip as a microfluidic platform format to prevent interconnections and dead volumes. This approach is clearly advantageous because it allows accurate, ultra-small volumes of samples (loop-size variable) to be introduced and has accurate fluid-control and manipulation capabilities.

Although the volume of research in this area is sizeable, real sample analysis is still in its infancy and constitutes one of the major challenges to microfluidics.[10] In addition, the current limitations and problem-solving strategies in quantitative analysis using microfluidic chips has very recently been revised, clearly indicating the significance of the matter and pointing out that the operators have to use even more care when creating their microchip-based analysis than they had done with conventional electrokinetic methods.[11]

On the other hand, microfluidic chips are emerging as an important alternative in food analysis in which a complicated sample preparation step is highly required.[12,13] One of the most important fields of food analysis is the accurate determination of extremely low levels of toxins and mycotoxins in foods.[14] Zearalenone (ZEA) is one mycotoxin which has attracted recent attention and its tolerable concentration in baby foods is under review because of the adverse effects it can have on human health.[15] Legal regulations have rapidly changed, lowering the minimum tolerable amounts in foods (ranging from 20 to 100 μg Kg^{-1}). Thus, it is of particular importance to develop sensitive analytical routes as anticipated security tools.[16,17] To be able to cope with the increasing number of sample matrices and mycotoxins of interest, fast and accurate analytical methods are needed. This demand has led to the development of rapid screening methods for single mycotoxins or whole mycotoxin classes based on immunochemical techniques (ELISA), biosensors and non-invasive optical techniques.[18–21] With respect to the determination of mycotoxins, another important aspect which has received attention[14] is the use of Certified Reference Materials (CRM). Although much has been done over the past few years regarding the production and certification of reference materials, there is still a need for further developments since these play a crucial role in the validation of new methods.

The literature on microfluidic immunosensing is growing and has recently been reviewed.[22,23] In addition, the use of microbeads has been exploited to improve the performance of the immunological reaction due to an increased surface-to-volume ratio, the assay kinetics are also being achieved more rapidly.[24,25] However, to the best of our knowledge, no microfluidic

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approaches have been reported on the control of mycotoxins in foods. In addition, none of these microsystems has used CRM to evaluate accuracy. In this paper, we are proposing the use of a microfluidic chip to control the regulatory limits (alarm levels) of ZEA in baby foods for the first time. Accuracy, using a CRM, the simplification of the calibration process, and the analysis of target samples are studied to demonstrate the analytical merits of this microfluidic approach.

Experimental

Chemicals and immunochemicals

Zearalenone (ZEA), bovine serum albumin (BSA), hydrogen peroxide (30%), hydroquinone (HQ), benzoquinone (BQN) and Tween 20 were purchased from Sigma-Aldrich. Standard ZEA solutions were prepared daily by diluting stock solutions (5 mg mL^{-1} in acetonitrile) in phosphate buffer saline (PBS). Anti-Zearalenone monoclonal antibody and the enzyme tracer ZEA conjugated to HRP were supplied by Soft Flow Biotechnology (Gödöllõ, Hungary). All of the other reagents were of the highest available grade.

Superparamagnetic polymer beads with protein G covalently coupled to the surface (Dynabeads® Protein G) were supplied by Invitrogen Dynal AS (Oslo, Norway).

Unless otherwise indicated, PBS or PBS modified with Tween and BSA was used. The composition of the PBS solution was phosphate buffer 10 mmol L^{-1}, pH 7.5 with 0.8% (w/v) NaCl. In the other PBS solution, 0.05% (v/v) Tween and 0.1% BSA were also added. Citrate-phosphate buffer (24.5 mmol L^{-1} citric acid and 51.7 mmol L^{-1} dibasic sodium phosphate), pH 5.0, was used for washing magnetic beads and the antibody capture procedure according to the manufacturer's protocol. All buffer solutions were prepared with Milli-Q water.

Samples

ZEA in maize certified reference material (83 ± 9 μg Kg^{-1}) was purchased from Sigma (BCR®). Powdered baby food (Blevit multicereales) containing a mixture of wheat, rice, maize, barley, oats, sorghum, rye, and soya flour was purchased from a local pharmacy (Madrid, Spain). Cereal milkshakes (Puleva) containing a mixture of wheat, rice, maize, barley, oats, and rye were purchased from a local retail store (Madrid, Spain).

Equipment

A single-channel glass microchip manufactured by Micralyne (Model MC-BF4-001, Edmond, Canada) using wet chemical etching and thermal bonding techniques was used. The microchip consisted of a glass plate (88 mm × 16 mm) with a four-way injection cross, a 74 mm longitudinal channel, and side arms measuring 5 mm. The original waste reservoir was cut off, leaving the channel outlet open at the end of the chip to facilitate end-channel amperometric detection. The channels were 50 μm wide and 20 μm deep. The glass chip was attached to the previously described microsystem.[8] The holder used contained both 100 μL of the sample reservoirs and a running buffer reservoir. Pipette tips were inserted into each of the three holes in the glass chip to permit solution contact between the channel and its corresponding reservoir on the chip holder. A platinum wire was inserted into each reservoir which served as a contact for the high-voltage power supply. The amperometric detector (on the end-channel side) consisted of an Ag/AgCl wire, used as the reference electrode, and a glassy carbon disk electrode (2 mm Ø) which was used as the working electrode. The working electrode was further held in place by a plastic screw which pressed the electrode against the channel outlet. Amperometric detection was performed using a Potentiostat Autolab PGSTAT 12 from Eco Chemie. The high-voltage power supply (from Glassman High Voltage Inc.) had an adjustable voltage range between 0 and +5000 V.

Magnetic separation during the washing steps was performed using a magnetic separation rack (Ohmicron). An ultrasonic bath from Sonic Vibra-Cell was used in the extraction procedure. The competitive curves were analyzed with a four-parameter logistic equation using suitable software (Graph Pad Prism 5).

Glassy carbon electrode cleaning

The electrode was cleaned before each session using three types of treatments: physical, chemical, and electrochemical. The physical treatment consisted of manually polishing the electrode with 1 μm and 0.05 μm alumina powder for 3 min each, and then rinsing them with deionized water. During the chemical treatment, the electrode was sonicated in ethanol solution for 15 s and then sonicated in deionized water for 15 s. The electrochemical treatment consisted of subjecting the electrode to cyclic voltammetry from −0.5 V to +1.5 v at a scan rate of 10 V/s for 15 min. The state of the electrode was then checked using 0.1 M $Fe(CN)_6^{4-}$ solution. This cleaning procedure was repeated after each analysis.

Microchip conditioning

Each of the glass microchip's channels were treated before use and between groups of runs by rinsing them with 0.1 M NaOH and deionized water for 15 min each. This procedure was carefully monitored to obtain reproducible results.

Immunoassay procedure

The immunoassay previously optimized was developed on the basis of a competition scheme, in which the mycotoxin zearalenone and an enzyme-labelled derivative compete for the binding sites of the specific antibody. A scheme of the followed route is shown in Fig. 1. Protein G covalently bound to magnetic particles acts as an oriented immobilisation support for the capture of the anti-mycotoxin antibody. After a molecular recognition event takes place, the extent of the affinity reaction is evaluated by the sensitive detection of an electrochemical mediator directly related to the activity of the enzyme tracer (HRP-zearalenone).

The competitive immunoassay was performed on single ELISA microplate wells that were adapted as holder reservoirs in the microfluidic device. After each incubation or washing step, the magnetic beads were separated from the supernatant by placing a magnet under the reservoir until the magnetic beads migrated to the bottom wall and the liquid, now clear, was then removed.

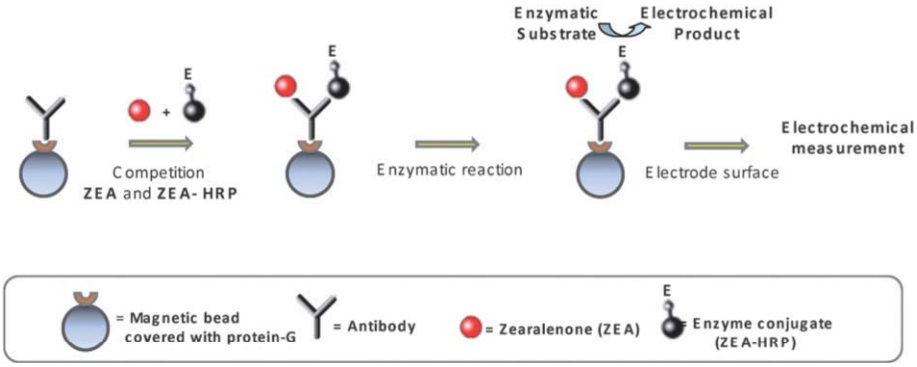

Fig. 1 Scheme of electrochemical immunoassay.

Initially, the Dynabeads® protein G magnetic particles were washed with citrate-phosphate buffer, pH 5.0, according to the manufacturer's protocol, in order to eliminate the storage buffer and to condition them for the IgG capture procedure. A volume of 2 μL of the beads was introduced into the well and, after precipitation of the beads by placing the well into the magnetic rack, the supernatant was removed. Then, 50 μL of specific antibody (5 μg mL^{-1}) prepared in citrate-phosphate buffer pH 5.0 was added to the beads. The solution was stirred gently for 30 min at room temperature to obtain antibody modified beads. The washing step was repeated five times with 200 μL of PBST which allowed any unbound antibody to be eliminated. The competitive assay was performed by re-suspending the antibody-coated beads in 50 μL of a mixture of either zearalenone standard solution or sample and the enzyme tracer (final dilution 1:200 in PBST-BSA 0.1%). The competitive immunological reaction was allowed to proceed with gentle stirring for 30 min at room temperature. The magnetic beads were again washed five times with 200 μL of PBST to eliminate any unbound species. Finally, Ag-Ab complex beads were re-suspended in 100 μL of solution containing the mediator (hydroquinone, HQ, 800 μM) and the enzymatic substrate (hydrogen peroxide, 600 μM). Hydrogen peroxide was used at this concentration to ensure that all enzymatic molecules were substrate bound and that Vmax (according to the Michaelis-Menten model) was reached. The enzymatic reaction was then allowed to proceed for 25 min, and supernatant was electrokinetically injected into the microfluidic platform where *end-channel* amperometric detection of the enzymatically oxidized mediator, HQ, took place.

Electrokinetic pumping procedure

The running buffer consisted of a solution of 10 mM PBS (pH = 7.5). The running buffer and the detection reservoirs were filled with PBS buffer. The sample reservoirs were either filled with the final solution obtained in the ELISA procedure containing the enzymatic reaction product, or the microELISA wells acted as sample reservoirs themselves, in which the enzymatic product appeared in the supernatant. A voltage of 2000 V was applied for 5 min to the buffer reservoir to fill the longitudinal channel, while the detection reservoir was grounded, leaving the other one floating. This procedure was also performed on each sample reservoir for 120 s to facilitate filling the injection channel (located between the longitudinal channel and the sample reservoir), and then voltage was applied for 5 min to the running buffer reservoir to eliminate the remains of any previously introduced samples from the longitudinal channel. Finally, the samples were injected by applying 2000 V for 7 s.

Amperometric detection

A detection of +0.1 V was applied to the working electrode. All experiments were performed at room temperature. A spacer (easily removable adhesive tape, 60 μm) between the surface of the electrode and the channel outlet was also used to control the distance between the electrode and the separation channel with reproducibility.

Safety considerations

The high-voltage supply should be handled with extreme care in order to avoid electric shock.

Extraction procedure

Solid samples (1 g) were extracted with 4 mL of acetonitrile:water (75:25, v/v) for 90 min at room temperature in an ultrasonic bath. Then, after centrifuging at 4000 rpm for 10 min, the supernatant was extracted and diluted one hundredfold in PBST-BSA. The diluted extracts were immediately assayed.

For evaluation purposes, solid baby food samples were spiked with known amounts of ZEA. Subsamples of 1 g were weighed and transferred to a centrifuge tube and directly spiked with a stock solution of ZEA in acetonitrile to a final concentration of 20 μg Kg^{-1}. The samples were allowed to equilibrate for at least 30 min before extraction. To asses accuracy, a maize certificate reference material (83 ± 9 μg Kg^{-1}) was determined. For the liquid samples (milkshakes containing cereal) the matrix effect was evaluated by determining undiluted samples spiked with a ZEA concentration of 0.83 μg L^{-1}. All of the experiments were analyzed in triplicate.

Results and discussion

The strategy presented to control the regulatory limits of ZEA in baby food products, based on the use of a microfluidic chip coupled to ELISA methodology, is inherently versatile: (i) in addition to its selectivity capabilities in immunoanalysis, microfluidic chips intrinsically allow the opportunity to govern

selectivity (the possibility of separating immunoassay products); (ii) a single-channel of a microfluidic chip can be used in a *multiplexed* format to perform methodological calibration and sequential analysis, dramatically reducing and simplifying the immunoanalysis protocol; (iii) furthermore, our microfluidic design allows a direct coupling of immunoassays and microfluidic chips since the external reservoirs of the microchip could be used as ELISA microwells in which only Pt electrodes chemically inert to immunoassays are required. In comparison with integrated approaches, this coupling strategy has the inherent advantages of not only easy fluidic manipulation and the possibility of increasing sensitivity *in situ* towards a suitable manipulation of the injection volume, but also the ability to implement it in all laboratories since clean-room facilities are not required.

Performance of the ELISA-microfluidic coupling system

An immunoassay procedure was performed on the external ELISA reservoir followed by the electrokinetic injection and pumping of the enzymatic product into the microfluidic chip to be detected. Fig. 2 shows the strategy proposed.

The electrochemical conditions were first studied. The driving voltages for the injection and pumping as well as injection times were assayed using a mixture of the electroactive products from the enzymatic reaction with the tracer: benzoquinone and hydrogen peroxide. A driving-pumping voltage of 2000 V with an injection time of 7 s was determined to be the optimal value. Under these electrokinetic conditions, the detection potential was studied. Fig. 3 shows the hydrodynamic voltamperograms obtained for both benzoquinone and hydrogen peroxide. As can be observed, a reduction in the benzoquinone began at a potential of +0.4 V, while hydrogen peroxide reduction started at negative potential below +0.1 V. In both cases, a plateau is observed at −0.1 V, corresponding to the diffusion limit zone. Therefore, selective detection of benzoquinone was accomplished at +0.1 V in which the microfluidic chip acted as an electrokinetic flow system.

Since the proposed immunoassay uses a competitive format, it is critical that the microfluidic chip be able to discriminate between the absence (maximum signal) and the presence of ZEA above regulatory limits (a clearly detectable decreased signal). Under the optimized parameters of the ELISA procedure, the critical ZEA concentrations were assayed in the microfluidic platform showing the expected signal profile, in which higher concentrations of the mycotoxin produced lower signal values. Thus, zearalenone concentrations of 0.83 and 10 ppb produced a reduction in the maximum signal (0 ppb ZEA) of up to 58.5% and 28.6%, respectively. Methodological calibration has to be constructed within the range of these limits and is usually performed by obtaining the typical four-parameter logistic curve fit. This is a highly time-consuming and laborious procedure. Due to the microchips' design (simple cross) which contains two reservoirs that can be used sequentially, it is possible integrate calibration and analysis of the mycotoxin on the chip itself. This strategy means that both reservoirs can be taken advantage of, in which one of them is used for calibration purposes and the other one is used to analyze the sample. In fact, the immunoassay procedure for an unknown zearalenone concentration (sample) takes place in the sample reservoir and the immunoassay procedure of a controlled amount of ZEA (control) takes place in the calibration reservoir (see Fig. 2). The enzymatic product of both reservoirs is sequentially injected and the amperometric signal is obtained. A new calibration factor for indirect determination was defined and calculated as $f_{calibration} = S_{ZEA\ standard} \times [ZEA]_{standard}$. Since this calibration factor is a constant for each analysis, ZEA concentration in the sample can be calculated as $[ZEA]_{sample} = f_{calibration}/S_{ZEA\ sample}$. The advantages are:

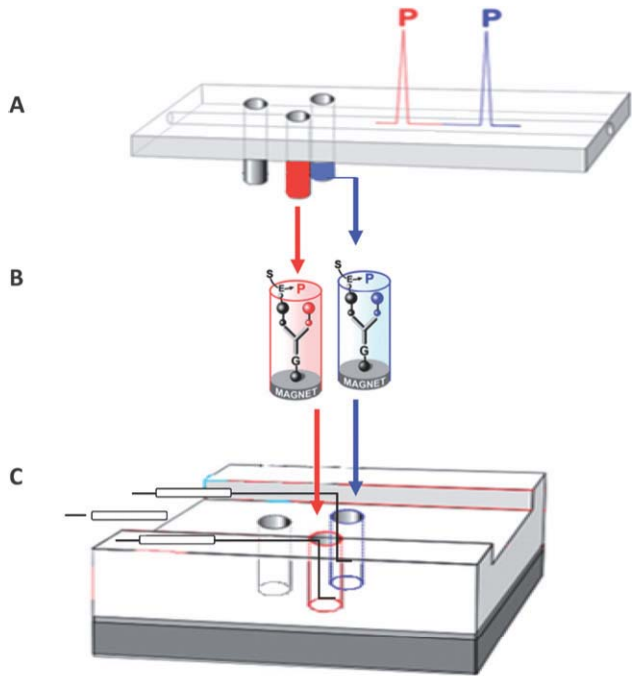

Fig. 2 Analytical strategy: (A) Microfluidic chip with internal reservoirs; (B) ELISA microwells; (C) External microchip reservoirs (left hand side for standard, right hand side for sample). For exact dimensions of microchip see Experimental section. P is the electroactive product to be detected, benzoquinone.

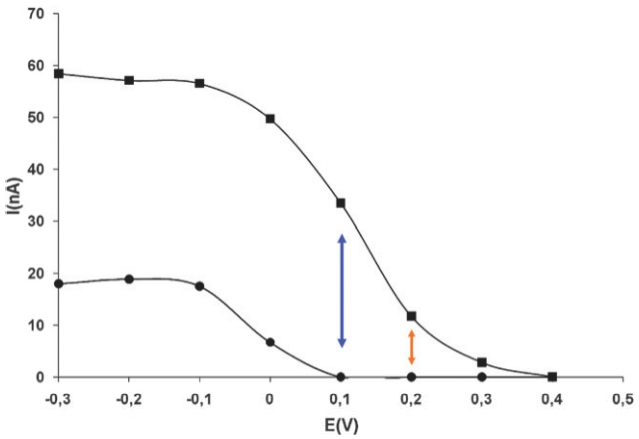

Fig. 3 The HRP enzyme is used as label and hydroquinone as a free mediator. HDVs for (●) hydrogen peroxide and (■) benzoquinone, performed on glassy carbon electrodes are presented. +0.1 V is found to be the optimal potential for selective detection of the benzoquinone.

(i) a dramatic simplification of the immunoanalysis calibration procedure, (ii) calibration and analysis are performed under equal conditions which helps to avoid other sources of error, and (iii) it is suitably designed, using the same concentration for the control as that expected for the sample, improving precision (and, subsequently, the accuracy and reliability) of the analysis (usually with the signal in the middle of the calibration graph where error is minimized close to the IC_{50} value).

Validation of the ELISA-microfluidic system

In order to demonstrate the analytical suitability of this proposal, the accuracy of the method was validated for a maize certified reference material (83 ± 9 μg Kg^{-1}). The diluted solution containing the mycotoxin from the CRM was assayed by the immunoassay procedure in the sample reservoir. Sequentially, a control solution of zearalenone of the same concentration as that expected in the diluted CRM was analyzed in the control reservoir. Enzymatic products as a result of the immunoassay were electrokinetically pumped sequentially and the detection of both signals was achieved following the strategy shown in Fig. 2. Fig. 4 shows the analytical signals obtained on the microfluidic platform that correspond to the integrated analysis of the CRM and the ZEA standard for three independent extractions, each measured in triplicate. A calibration factor was calculated in each case and used to perform the quantisation. In Table 1, excellent agreement of the results obtained within the uncertainty of the analyzed CRM can be observed along with excellent accuracy and reproducibility. The results demonstrate the suitability of the microfluidic platform as an analytical alternative to the conventional calibration procedure, simplifying the overall process. To the best of our knowledge, this is the first time that a high quality standard such as a CRM is used to evaluate the performance of microfluidic chips in the field of real sample analysis. This fact indicates the suitability of the proposed microchip strategy and constitutes an important finding in the analytical metrology of these microsystems as well.

Table 1 Validation of the ELISA-microfluidic system. Analysis of a corn CRM

Extraction	$[ZEA]_{Measured\ CRM}$ (ppb)	$[ZEA]_{Real\ CRM}$ (ppb)
1	75 ± 1	83 ± 9
2	79 ± 9	
3	77 ± 4	

Determination of zearalenone in baby food samples

Regulatory limits of ZEA in baby food samples were also analyzed by the microfluidic immunoassay. Liquid samples (milkshakes with cereal) and solid samples (powdered baby food containing different cereals) were analyzed as an example of different types of food matrices.

Since immunoassay implies a dilution step, measurement of the final concentration is critical. Therefore, since ZEA has to be extracted from solid samples, these samples were studied first. A powdered baby food was analyzed using the microfluidic approach according to the integration strategy. The sample, which was previously determined to contain an undetectable level of ZEA (Fig. 5A), was spiked at the lowest level permitted, or 20 ppb, of the target mycotoxin (Fig. 5B). Extraction and dilution was carried out according to the experimental procedure to reach a final concentration of about 1 ppb (in our case 0.83 ppb).

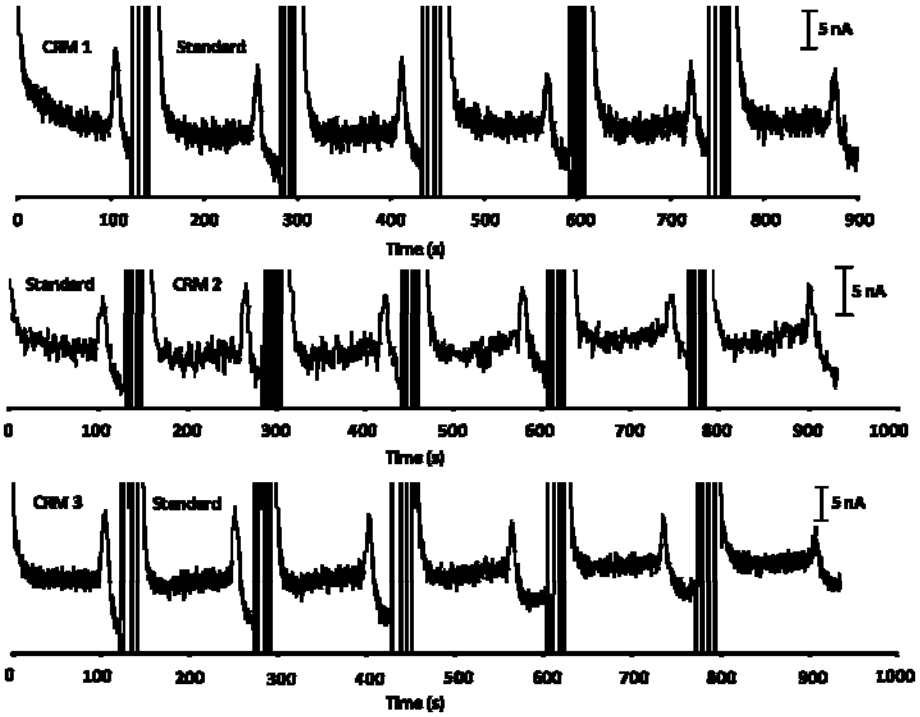

Fig. 4 Amperometric signals of CRM sample and standard replicates using integrated calibration "on-chip".

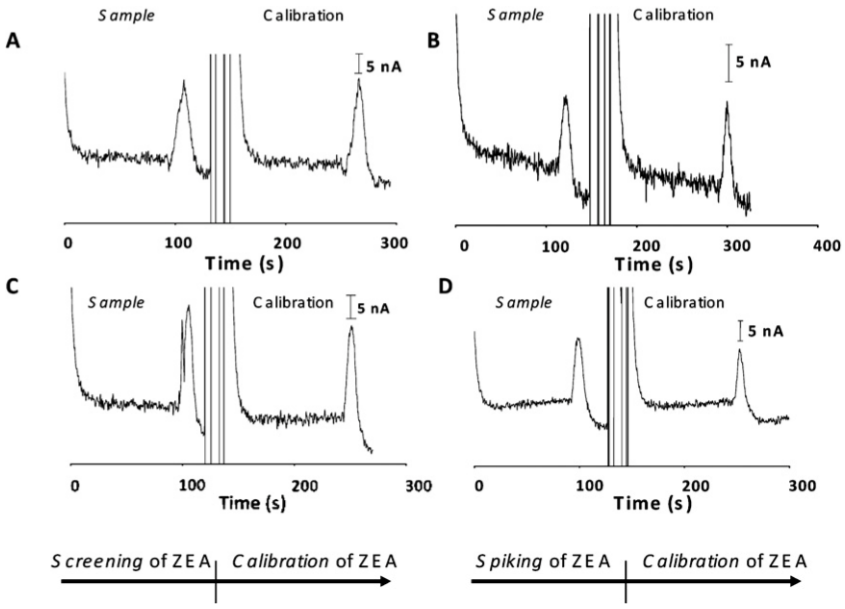

Fig. 5 Analysis of baby food samples. Solid samples (A and B): powdered cereal baby food. Liquid sample (C and D): cereal milkshake. (A) Screening of ZEA in powdered cereal baby food and (B) spiking of ZEA at 20 ppb in powdered cereal baby food. (C) Screening of ZEA in milkshake and (D) spiking of ZEA at 0.83 ppb in powdered cereal baby food.

In most liquid samples, however, the extraction procedure can be saved, though matrix interferences by lipids or proteins in the sample can disrupt the interaction between antigen and antibody. In this paper, undiluted cereal milkshakes were determined without sample pre-treatment to explore the presence of the ZEA mycotoxin. Using the integrated calibration microfluidic immunoassay, a milkshake sample ([ZEA] not added) was analyzed as shown in Fig. 5C. After the sample was found to contain an undetectable level of the mycotoxin, it was spiked with about 1 ppb (0.83 ppb) of zearalenone (Fig. 5D), since this was the ZEA level measured in the solid sample (well below European directive requirements).

Table 2 depicts the quantitative analysis for baby food samples with the integrated calibration approach. The calibration factor was also calculated for each analysis. Excellent recoveries were obtained for both samples, for both limits during the control stage for the absence of ZEA (direct analysis of the sample), and the presence of ZEA remained under the allowable legal levels (20 ppb) even when extremely low values (1 ppb) were checked.

Table 2 Control of zearalenone concentration levels in infant food using the integrated calibration approach

	Recovery (%)	
[ZEA] (ppb)	Liquid sample	Solid sample
0	101 ± 7	104 ± 3
0.83[a]	95 ± 4	92 ± 2

[a] Final concentration measured in the samples. In the case of the solid sample, it was initially spiked with 20 ppb although after extraction and dilution the final concentration is 0.83 ppb.

Additional features

The microfluidic approach developed allows additional possibilities. Indeed, a direct adjustment of the different immunoassay steps is possible due to selective manipulation of the fluids inside of the microchip. In this sense, a reduction in the incubation time of the enzyme substrate with the tracer can be accomplished as a result of increasing the electrokinetic injection volumes. Different incubation times (25, 15, and 5 min) were assayed, maintaining a constant electrokinetic injection time of 7 s. Different injection times (7, 10, and 13 s) were also assayed maintaining a constant incubation time of 15 min. Table 3 lists the results obtained. It was observed that an optimal response could be obtained with a 15 min incubation time (enzyme-substrate) and 13 s of electrokinetic injections or with a 25 min incubation time and an injection time of 7 s (indicated in grey colour). As expected, the microfluidic approach allowed sensitivity to be manipulated *in situ*.

In order to achieve a robust coupling of ELISA and microfluidic chips, the possibility of injecting the enzymatic products directly from the ELISA reservoir onto the microfluidic chip was also explored. A homemade modification was carried out incorporating Pt wires in ELISA reservoirs to act as external microchip reservoirs. After the immunoassay procedure takes places inside the ELISA microwell, a magnet was placed under the reservoir to precipitate the magnetic beads and consecutive injections were then performed. RSD for amperometric currents obtained in repetitive injections was 9.2% ($n = 6$) indicating the stability of the signal and, therefore, that the enzymatic reaction was stopped at least in the supernatant of the ELISA reservoir. In addition, comparison with a control experiment performed in the different ELISA reservoirs (with aliquot transfer to the microfluidic reservoir prior to injection) reveals excellent reproducibility, with a RSD = 9.2% ($n = 5$), showing that both

Table 3 Flexibility of the proposed ELISA-microfluidic coupled device.[a] The optimum values are highlighted in bold

Injection time (s)	Incubation time (min)	Analytical signal (nA)	Incubation time (min)	Injection time (s)	Analytical signal (nA)
7	**25**	**16 ± 1**	**15**	7	8.9 ± 0.9
	15	8.9 ± 0.9		10	14.5 ± 1.5
	5	5.8 ± 0.3		**13**	**16.4 ± 1.6**

[a] Values are expressed as Mean values ± standard deviation ($n = 3$).

working formats were equally suitable. This opens a new possibility for future coupling of ELISA and microfluidic chips in a multiplexing configuration.

Conclusions

Anticipation surrounds microfluidic chips as useful new tools to reliably control extremely low levels of ZEA below legislation in baby food products. This platform allows ZEA to quickly be detected in real samples of special concern using simplified calibration in under 200 s with accuracy. Additionally, microfluidic chips allow external ELISA to be directly coupled, making it an attractive option as a new tool for food safety because of their speed, extremely low sample volume requirements, calibration simplicity, and their versatility to be manipulated, avoiding complex integration approaches but benefiting from the inherent benefits of miniaturization. In consequence, the future design and fabrication of a multichannel chip for multiplexed analysis of mycotoxins is under construction in our minds.

Acknowledgements

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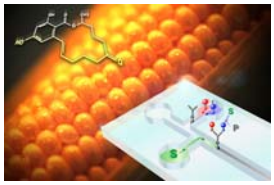
III.5 ARTÍCULO 4:



Integración total de un método ELISA electroquímico en una plataforma microfluídica para la detección y control de niveles de ZEA en alimentos infantiles.

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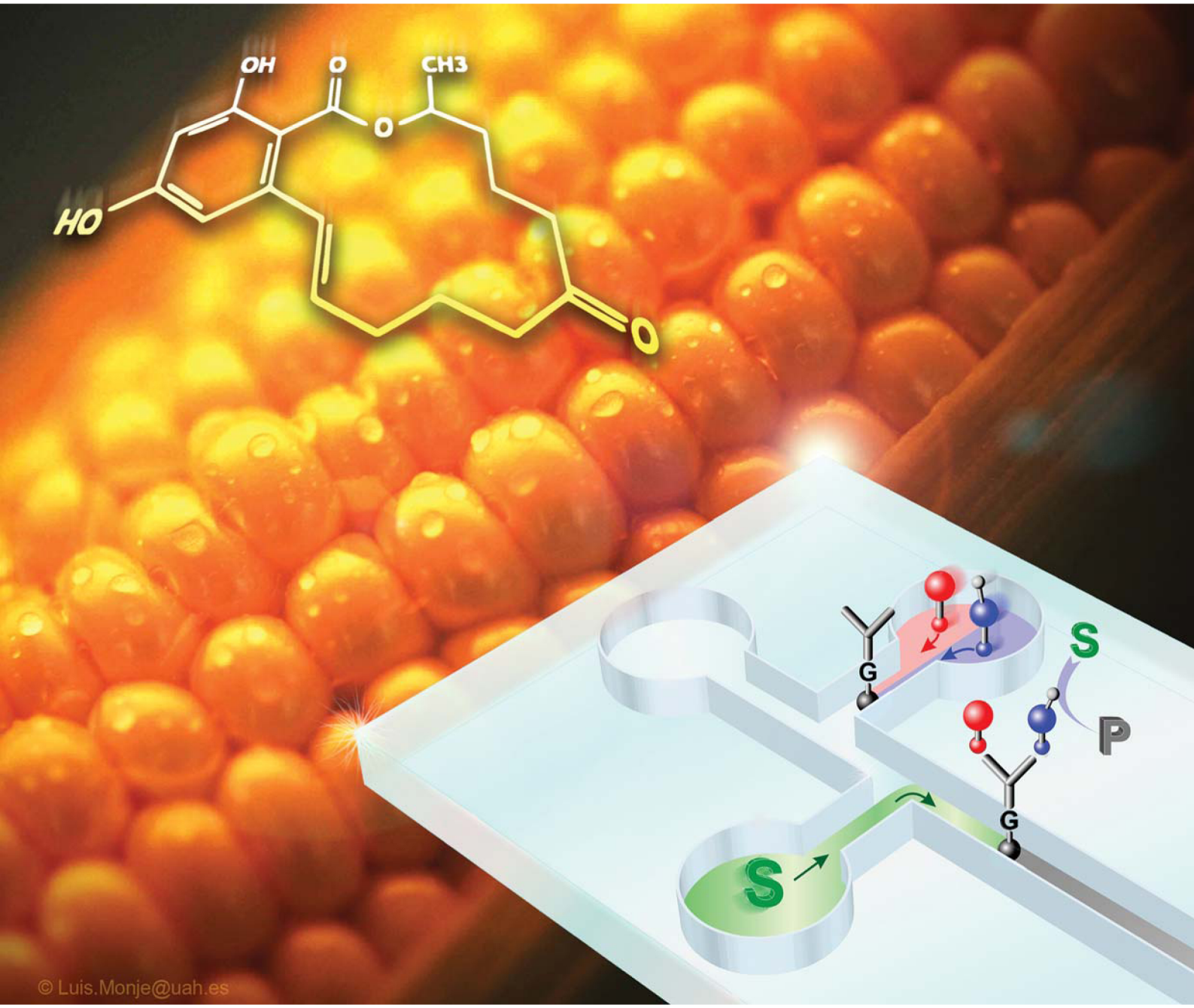

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HOT ARTICLE

Alberto Escarpa *et al.*
Integrated electrokinetic magnetic bead-based electrochemical immunoassay on microfluidic chips for reliable control of permitted levels of zearalenone in infant foods

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PAPER

Integrated electrokinetic magnetic bead-based electrochemical immunoassay on microfluidic chips for reliable control of permitted levels of zearalenone in infant foods†

Mirian Hervás, Miguel A. López and Alberto Escarpa*

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Microfluidic technology has now become a novel sensing platform where different analytical steps, biological recognition materials and suitable transducers can be cleverly integrated yielding a new sensor generation. A novel "lab-on-a-chip" strategy integrating an electrokinetic magnetic bead-based electrochemical immunoassay on a microfluidic chip for reliable control of permitted levels of zearalenone in infant foods is proposed. The strategy implies the creative use of the simple channel layout of the double-T microchip to perform sequentially the immunointeraction and enzymatic reaction by applying a program of electric fields suitably connected to the reservoirs for driving the fluidics at different chambers in order to perform the different reactions. Both zones are used with the aid of a magnetic field to avoid in a very simple and elegant way the non-specific adsorption. Immunological reaction is performed under a competitive enzyme-linked immunosorbent assay (ELISA) where the mycotoxin ZEA and an enzyme-labelled derivative compete for the binding sites of the specific monoclonal antibody immobilised onto protein G modified magnetic beads. Horseradish peroxidase (HRP), in the presence of hydrogen peroxide, catalyses the oxidation of hydroquinone (HQ) to benzoquinone (BQN), whose back electrochemical reduction was detected at +0.1 V. Controlled-electrokinetic fluidic handling optimized conditions are addressed for all analytical steps cited above, and allows performing the complete immunoassay for the target ZEA analyte in less than 15 minutes with unique analytical merits: competitive immunoassay currents showed a very well-defined concentration dependence with a good precision as well as a suitable limit of detection of 0.4 μg L^{-1}, well below the legislative requirements, and an extremely low systematic error of 2% from the analysis of a maize certified reference material revealing additionally an excellent accuracy. Also, the reliability of the approach is demonstrated by the analysis of selected infant foods yielding the strictest ZEA permitted levels and excellent recoveries of 103 and 101% for solid and liquid samples, respectively.

1. Introduction

In the last decade, the combination of microfluidic technology and immunoassays has been proven as an emergent and powerful alternative in the analysis of a broad variety of analytes of clinical, food industry and environmental significance. Consequently, numerous research achievements have already been reported based on the development of microfluidic immunosensors that combine the analytical power of microfluidic devices with the high sensitivity and specificity associated with the antigen–antibody interactions.[1–3] Microchip platforms can dramatically improve the immunosensing performance by decreasing analysis time, reducing the consumption of reagents and samples, increasing the reliability and sensitivity through automation, integrating multifunctional components and process in a single device, as well as the potential of parallelisation of analyses into a small monolithic piece. In addition, microfluidics and microchip technology allow, on a single (monolithic) piece, the integration of biological recognition event and chemical transduction by fluidic handling in a very unique way yielding a new sensor generation. To achieve the aim of successful construction of a microfluidic immunosensor, several aspects must be considered. Mainly, the materials and microfabrication methods, fluid handling, antibodies/antigens immobilisation and detection schemes.

The design complexity and materials used for microchip immunosensors are quite different depending on the

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applications, although the majority of them are based on glass, silicon and polymers. Glass and silicon were the predominant materials in the initial works since the microfabrication technologies such as patterning, etching and bonding were well established in the semiconductor industry. Even today those approaches are used although most of the time in combination with polymers.[4–9] Lately, polymer has emerged as the chosen material due to economy (only expensive patterning is required for constructing the mold) and variety of fabrication procedures (hot embossing, soft lithography, laser photoablation, screen printing).[10–20] These different fabrication possibilities and the feasibility of polymer materials have allowed the production of sophisticated designs in contrast with those more rudimentary ones produced in glass.

Alternatively, different fluid handling procedures have been reported in the literature. Hydrodynamic pumping is without a doubt the most used method although in most cases syringe pumps are used to control the flow of the liquid solutions. Microfabrication of valves and pumps, besides being difficult and expensive to miniaturise, requires clean room facility technology.

On the other hand, the use of electrokinetic-EOF based fluidic motivation benefits "lab-on-a-chip" devices because the flow in multiple channels on a microchip can easily be controlled with a few electrodes. This further simplifies the apparatus by eliminating valves and pumps where dead volumes are zero and band broadening is reduced yielding a better pumping efficiency. In addition, in comparison with hydrodynamics, electrokinetics offer very important valuable features for "lab-on-a-chip" (bio-)immunosensing devices since it allows us to modify the injected amounts of antibody, antigen, enzymatic tracer and substrate used in the different analytical steps by simply changing the electric field and time applied (acting as a variable well-controlled loop) as well as manipulating the fluidics in different directions including reversed and stopped flows with extreme simplicity and automation. These advantages become potentially very attractive for the development of microfluidic immunosensors since competitive immunoreactions and enzymatic reaction require integration in different zones of the microfluidic platform under different electrokinetic conditions (injection, amounts, flow rates). Electrokinetic motivation[21–23] together with other attempts like electrowetting,[4] finger pressure[24,25] or centrifugal force[26] has already been used for liquid displacement.

Immobilisation of the capture antibody (or antigen) onto an adequate solid support within the microdevice is a key parameter in the construction of the immunosensor. An inadequate immobilisation method could result in either low bioactivity of the probes or high background (non-specific adsorption). Different approaches have been presented in the literature, such as immobilisation directly onto the microchannel surface,[8,15,18,20,27,28] onto the electrode surface inside the channel,[4,7,11,12,25] sol–gel matrix,[13] membranes[14] or using free-flow reactions in affinity capillary electrophoresis.[21,23] However, microbeads are the most popular support for antibody immobilisation. These beads are made of different materials such as paramagnetic core,[5,9,16,29–33] polystyrene,[6,19,34–37] methacrylate,[38] silica,[39,40] agarose,[41] and control pore glass,[26,42–45] and the aid of magnetic fields or physical trapping can be used to manipulate them within the microchannels at different stages of the assay. Bead-based microfluidic immunoassays have some advantages compared to normal fluidic systems and microtiter wells used in ordinary immunoassays. Those include an increased surface to volume ratio, while diffusion distances in microchannels are reduced. This results in higher sensitivity of assays due to high efficiency of interactions between samples and reagents. Easy manipulation and transport of the beads in a fluidic system and a variety of surface modifications available on these microbeads allow an easy way for the biomolecules to be attached.

Furthermore, the detection scheme is another significant factor in the development of microfluidic immunosensors. Although fluorescence,[9,14,15,20,31–36,38–40] mainly laser-induced fluorescence (LIF), is the detection route most used because of its inherent sensitivity, other strategies have been applied for on-chip, immunoassays. Some examples are reported in the literature where chemiluminiscence,[23] optical adsorption,[46] thermal lens microscopy[47] or mass spectrometry[48] are also used. However, electrochemical detection has been proven as an ideal and valuable technique to be incorporated in miniaturised devices, due to its inherent facility for miniaturisation without loss of performance, high sensitivity and compatibility with microfabrication techniques.[4–8,11–13,16,18,19,26–30,37,41–45] On the other hand, food analysis is an analytical field where microfluidic chips have emerged as an important tool for complicated samples where a pretreatment step is highly required.[49,50] In the last few years, a great effort has been devoted to the accurate determination of extremely low levels of toxins and mycotoxins in food.[51] Mycotoxins are secondary metabolites produced by different fungi present in agricultural commodities. They grow under a wide range of climatic conditions in the field and during storage. Fungal contamination is a real problem, not only by the economical aspects related to the industries but also by the significant health risks to humans and animals that consume contaminated feeds.[52]

Zearalenone (ZEA) (6-[10-hydroxy-6-oxo-*trans*-1-undecenyl]-β-resorcyclic acid lactone) is a non-steroidal estrogenic mycotoxin produced by several species of *Fusarium*. These fungi colonise different cereals such as maize, barley, oats, wheat, rice and sorghum in temperate and warm countries.[53] ZEA is known to cause estrogenic effects at relatively low levels in pigs, sheep, other farm animals and humans. Those effects include infertility, reduced serum testosterone levels and sperm counts, reduced incidence of pregnancy and change in progesterone levels, and it has been associated with hyperplasic and neoplasic endometrium and human cervical cancer.[54] Legal regulations have specified the maximum levels of ZEA in different commodities. A maximum tolerable amount of 20, 75 and 100 μg kg^{-1} has been set in baby food, cereal flour and unprocessed cereals, respectively.[55–57] Therefore, it is of particular importance to develop sensitive and efficient analytical tools for the determination of ZEA in food samples.

Several analytical methods for the determination of ZEA have been reported in the literature, including thin-layer chromatography, gas chromatography, high-performance liquid chromatography with UV, fluorescence or mass spectrometry detection, capillary electrophoresis, direct electrochemical analysis and enzyme-linked immunosorbent assay (ELISA). However, to the best of our knowledge, only a few examples of microfluidic immunosensor approaches have been reported on

the control of mycotoxins or fungi in foods.[58,59] In this case they were non-integrated hydrodynamic approaches.

In this article we report a total integrated microfluidic immunoassay into the microchannels of a simple layout of double-T glass microchip. Magnetic beads act as an antibody immobilisation support which can be easily handled and retained by the action of an external magnet. The microfluidic platform integrates all required immunoassay steps where different reagents and the sample are automatically moved following a predefined potential and time program by electrokinetic injection and pumping. Immunoassay was carried out using a competitive immunoassay where the sample is allowed to compete with a ZEA–HRP conjugate for the immobilised antibodies. Immunological complex formation and enzymatic reaction are physically separated in different channels avoiding the problem of non-specific adsorption usually associated with these devices. The detection scheme is based on the use of HRP enzyme which in the presence of hydrogen peroxide catalyses the oxidation of hydroquinone (HQN) to benzoquinone (BQN). The electrochemical reduction back to HQN was detected at 0.1 V. The response current obtained from the product of enzymatic reaction is proportional to the activity of the enzyme and, consequently, to the amount of ZEA mycotoxin in the sample. The capabilities of the system have been demonstrated in terms of accuracy, precision and by reliable ZEA determination in infant food samples concerned.

2. Experimental

2.1 Materials and methods

Zearalenone (ZEA), bovine serum albumin (BSA), hydrogen peroxide (30%), hydroquinone (HQ), benzoquinone (BQN), Tween 20 and other buffer reagents were purchased from Sigma-Aldrich (Madrid, Spain). Standard ZEA solutions were prepared daily by diluting the stock solution (5 mg mL^{-1} in acetonitrile) in phosphate buffer saline (PBS). Anti-ZEA monoclonal antibody and the enzyme tracer ZEA–HRP (horseradish peroxidase) conjugate were supplied by Soft Flow Biotechnology (Gödöllö, Hungary).

The composition of the PBS solution was phosphate buffer 10 mmol L^{-1}, pH 7.4 with 0.8% (w/v) NaCl. In PBS–T and PBS–T–BSA, 0.05% (v/v) Tween and 0.1% BSA were also added. Citrate–phosphate buffer, pH 5.0, composition was: 24.5 mmol L^{-1} citric acid and 51.7 mmol L^{-1} dibasic sodium phosphate. All buffer solutions were prepared with water obtained by a Milli-Q system.

Superparamagnetic polymer beads with protein G covalently coupled to the surface (Dynabeads® Protein G) were supplied by Invitrogen Dynal AS (Barcelona, Spain).

Samples. ZEA in maize certified reference material (83 ± 9 μg kg^{-1}) (ERM®BC717) was purchased from IRMM. Powdered baby food (Blevit multicereales) containing a mixture of wheat, rice, maize, barley, oats, sorghum, rye and soya flavour was purchased from a local pharmacy (Madrid, Spain). Cereal milkshakes (Puleva) containing a mixture of wheat, rice, maize, barley, oats, and rye were purchased from a local retail store (Madrid, Spain).

Equipment. A double-T channel geometry glass microchip manufactured by Micralyne (model MC-BF4-TT100, Edmonton, Canada) using wet chemical etching and thermal bonding techniques was used. The microchip consisted of a glass plate (88 mm × 16 mm) where the total length of the separation channel is 85 mm with a semicircular section of 50 μm width and 20 μm depth. The joint formed by injection (5 mm side arms) and separation channels has the shape of a double-T. The original waste reservoir was cut off, leaving the channel outlet open at the end of the chip to facilitate end-channel amperometric detection. The glass chip was attached to a Plexiglas holder containing both 100 μL sample reservoirs and a running buffer reservoir. Pipette tips were inserted into each of the three holes in the glass chip to permit solution contact between the channel and its corresponding reservoir on the chip holder. A platinum wire was inserted into each reservoir which served as a contact for the high-voltage power supply. The amperometric detector located at the end of the longitudinal channel on the holder detection reservoir and following a wall-jet end channel configuration[22,60] consisted of an Ag/AgCl wire, used as a pseudoreference electrode, platinum wire as counter and a glassy carbon disk electrode (2 mm diameter) which was used as the working electrode. These wire (reference and counter) electrodes were inserted through holes drilled in the Plexiglas holder. The working electrode was further held in place by a plastic screw which pressed the electrode against the channel outlet. Amperometric detection was performed using a potentiostat Autolab PGSTAT 12 from Eco Chemie. LabSmith HVS448 High Voltage Sequencer with eight independent high-voltage channels and programmable sequencing for an entire level of voltage manipulation (LabSmith, Livermore, CA) was used as voltage source. A neodymium disk magnet (22.0 mm diameter, 10.0 mm height) was used to handle and retain the magnetic beads. An ultrasonic bath from Sonic Vibra-Cell was used in the extraction procedure.

Competitive curves were analysed with a four-parameter logistic equation using suitable software (GraphPad Prism 5).

Glassy carbon electrode cleaning. The electrode was cleaned before each session using three types of treatments: physical, chemical, and electrochemical. The physical treatment consisted of manually polishing the electrode with 1 μm and 0.05 μm alumina powder for 3 min each, and then rinsing them with deionised water. During the chemical treatment, the electrode was sonicated in ethanol solution for 15 s. The electrochemical treatment consisted of subjecting the electrode to cyclic voltammetry from −0.5 V to 1.5 V at a scan rate of 10 V s^{-1} for 15 min. The state of the electrode was then checked using 10^{-4} M $[Fe(CN)_6]^{4-}$ solution.

Microchip conditioning. Each of the glass microchip's channels was treated before by rinsing them with deionised water and 0.1 M NaOH for 10 and 30 min respectively. This procedure was carefully monitored to obtain reproducible results.

Immunoassay procedure. The immunoanalytical methodology was developed on the basis of a competition scheme where the mycotoxin ZEA and an enzyme-labelled derivatives compete for the binding sites of the specific monoclonal antibody. Magnetic beads covered with covalently bounded protein G were used as

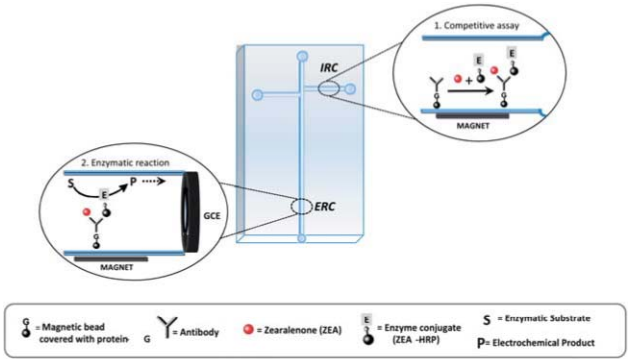

Fig. 1 Microfluidic layout and immunoassay principle (IRC = immunological reaction chamber and ERC = enzymatic reaction chamber).

an oriented immobilisation support for the capture of the anti-ZEA antibody. After the molecular recognition event, the extent of the affinity reaction was evaluated by the addition of the enzymatic substrate and electrochemical mediator. Its reduction on the electrode surface was directly related to the activity of the enzyme tracer (HRP–ZEA) and hence, inversely corresponded to the mycotoxin concentration. Fig. 1 shows the electrochemical immunoassay principle on microfluidic chip layout used.

Antibody immobilisation onto the protein G modified magnetic beads. The procedure for antibody immobilisation onto the magnetic beads was always performed on single ELISA microplate wells, *off-chip*. In this sense, a volume of 2 μL of magnetic beads coupled with protein G were washed with citrate–phosphate buffer, pH 5.0, according to the manufacture protocol in order to eliminate the storage buffer and condition them for the IgG capture procedure. Then, 50 μL of specific antibody (5 μg mL^{-1}) prepared in citrate–phosphate buffer, pH 5.0, was added to the volume of Dynabeads. The solution was stirred gently for

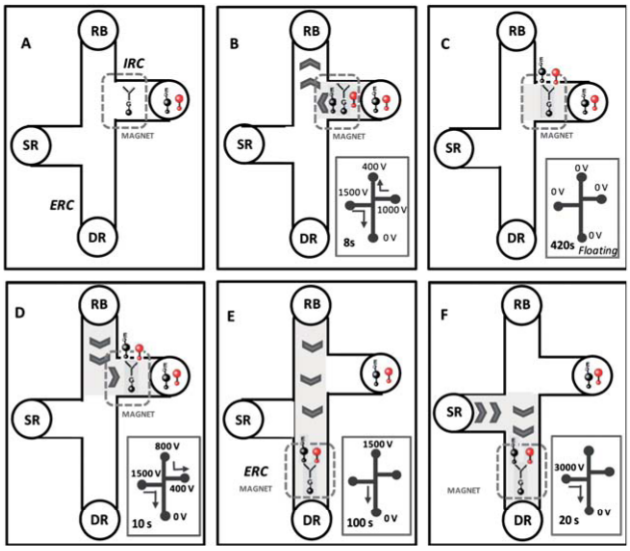

Fig. 2 Detailed schematic representation of the different analytical and electrokinetic (insets) stages of the electrochemical microfluidic immunosensing strategy for zearalenone detection (RB: running buffer reservoir; SR: substrate reservoir; DR: detection reservoir; IRC: immunological reaction chamber, and ERC: enzymatic reaction chamber).

30 min at room temperature to obtain antibody-modified beads. A washing step with 200 μL of PBS–T was repeated five times to ensure elimination of the non-bound antibody. After each washing step, the magnetic beads were separated from the supernatant by placing a magnet under the reservoir until the magnetic beads migrated to the bottom wall and the liquid, now clear, was then removed. Modified beads were re-suspended in 50 μL PBS and kept at 4 °C until use. Concentration and incubation time of the antibody were used as previously optimised.[61] Immobilised antibody preparations were perfectly stable for at least 1 month.

Immunoassay procedure into the microfluidic device. The analytical and electrokinetic steps of electrochemical microfluidic immunoassay are shown in Fig. 2. Antibody-modified magnetic beads (0.2 μL suspension) were introduced and retained in a fixed position of the immunological reaction chamber (IRC) by the action of a magnetic field generated by a removable external magnet (Fig. 2A). Afterwards, all the stages were performed within the microchip and reagents and sample were electrokinetically injected and pumped automatically by the adequate programming of the high-voltage power supply. Competition was then performed by injection and pumping a mixture of either ZEA standard solution or sample and the enzyme tracer (1 : 200 in PBS–T–BSA 0.1%) from the sample reservoir through the beads bed (Fig. 2B). To achieve this goal, a combination of potentials was applied to the different reservoirs for 8 s. Then, the competitive immunological reaction was allowed to proceed stopping the flow. For this purpose, all reservoirs were held under floating conditions (no potentials were applied) for 420 s (Fig. 2C). After that, a washing step of the immunological reaction chamber took place. In this sense, a reverse flow with PBS directs the reagents back towards the reservoir, eliminating any non-bound species. The corresponding combination of potentials was applied for 10 s (Fig. 2D). For the next step, the beads were dragged with the magnet to the enzymatic reaction chamber (ERC) where enzymatic reaction would proceed. At this point, an additional washing step of the beads carrying the immune complex was done with PBS along the central channel for 100 s (Fig. 2E). Enzymatic reaction was carried out by injection and pumping from the substrate reservoir a mixture of hydrogen peroxide (1.2 mM) and hydroquinone (1.6 mM) through the beads for 20 s (Fig. 2F). The electrochemical detection of the oxidised mediator (benzoquinone) was accomplished downstream at 0.1 V end-channel configuration. The stock solution of hydroquinone was prepared freshly before the experiment.

Sample extraction procedure. Solid samples (1 g) were extracted with 4 mL of acetonitrile : water (75 : 25, v/v) for 90 min at room temperature in an ultrasonic bath. Then, after centrifugation at 4000 rpm for 10 min, the supernatant was extracted and diluted in PBS–T–BSA to minimise the influence of the organics solvents.

3. Results and discussion

In this work we have designed a strategy which implies the creative definition and use of the simple double-T microchip

geometry in order to perform the complete electrochemical immunoassay. The strategy was based on the use of the two channels of its layout: injector and separation channels to perform the immunocompetition and the enzymatic reactions, respectively. The complete analytical strategy is shown in Fig. 2. Indeed, the injector arm has been termed the immunological reaction chamber (IRC), and it is the microchannel where the immunological complex formation between antibody-coated beads and sample or enzyme-conjugate takes places (Fig. 2A, B, C and D). For the second stage, the modified beads were dragged to a region of the longitudinal channel near the detector called the enzymatic reaction chamber (ERC) by the aid of a removable magnet. In this area, the enzymatic product is obtained from the antibody-retained HRP-conjugate and then detected (Fig. 2E and F). This approach is based on physical separation of both zones avoiding, in an elegant way, one of the greatest problems associated with microfluidic immunoassays as nonspecific adsorption to the channel walls surfaces. The use of magnetic beads that can be easily handled and double-T geometry of the chip was especially appropriate for this configuration. Once the layout-based strategy was defined, optimisation of the different stages in both areas of the microfluidic immunoassay was performed.

3.1 Optimisation of the experimental variables related to enzymatic reaction and electrochemical detection

In a first step, only the enzymatic reaction and product detection within the microfluidic device were optimised. In consequence and as a preliminary step, only the immunological competition event was previously performed in ELISA wells, *off-chip*. After this process, the tracer–antibody complex modified magnetic beads were hydrodynamically introduced into the microchip and retained with the aid of the magnet at the ERC. Different parameters affecting the enzyme reaction and detection within the microchip were then studied.

Firstly, the volume of modified magnetic beads introduced into the microfluidic device was assayed. A higher amount of beads could imply a higher signal, given that a higher amount of enzyme conjugates can be captured by the antibody. However, their physical position within the channel could affect the physical availability of the enzyme and, even more, they can obstruct the flow through the channel. A range of 0.1 to 2 μL from the resuspended 50 μL modified beads was studied where only enzyme-labelled derivatives compete for the binding sites of the antibody (Table S1†). The highest signal was obtained for 0.2 μL, while higher amount of beads rendered noise and broader peaks up to 2 μL where the peak was almost distorted. Finally a bead volume of 0.2 μL was used to evaluate other parameters.

Secondly, horseradish peroxidase enzyme-conjugate activity was optimised by the addition of hydrogen peroxide as substrate and hydroquinone as electrochemical mediator. The HRP, in the presence of hydrogen peroxide, catalyses the oxidation of hydroquinone (HQ) to benzoquinone (BQN) whose back electrochemical reduction was detected at +0.1 V. The amperometric current was assayed for different concentrations ranging between 0.6 to 1.8 mM for H_2O_2 and 0.8 to 2.4 mM HQ. Maximum signal was achieved for 1.2 mM hydrogen peroxide and 1.6 mM HQ, reaching a plateau where higher concentrations did not render higher signals. Therefore these concentrations were selected for further measurements.

One of the beauties of electrokinetic motion is the easy manipulation of fluids within the microchip. In this sense, electrokinetic injection time and potential applied govern the amount of substrate and electrochemical mediator injected. To get adequate amounts, potentials from 1500 V to 3000 V and injection time within a range of 10–80 s for each potential were assayed. The highest signal was obtained for the combination of 3000 V and 20 s (Table S2†).

The flow rate of reagents solution passing through the microfluidic channels is a critical factor. In this sense, the flow rate of substrate and redox mediator through the microchip channel, where the beads bed is located, affect the yield of enzymatic reaction and response of the electrochemical detector. In this work, the flow rate is controlled by electrokinetics towards the applied potential. The optimal flow rate was determined by evaluation of the generated current after enzymatic reaction when reagents are pumped at different driving potentials from 1000 to 1750 V. The signal profile showed a slight increment up to 1500 V, followed by a decrement for next potentials. Taking into account the size of the response and even the analysis time for each potential value tested, 1500 V was chosen as driven potential in further experiments (Table S3†). A detection potential of +0.1 V, using glassy carbon electrode positioned at end-channel configuration *vs.* Ag/AgCl reference electrode and platinum wire as counter, was used as reported previously from hydrodynamic voltammograms.[22]

3.2 Optimisation of the experimental variables related with immunological interaction

Once the signal production was optimised, the best conditions for immunological interactions within the microchip were then studied.

The microfluidic device was initially prepared by hydrodynamical introduction of 0.2 μL antibody-modified beads into the IRC. Competition between sample and ZEA–enzyme conjugate for binding to the beads could then be performed within the arm-side channel. In this sense, a mixture of ZEA standard or sample and the enzyme tracer was electrokinetically injected and allowed to react with the immobilised antibody following the scheme presented in Fig. 2B. As injection time and driving potential control the sample and tracer volume to react with the immobilised antibody, these variables were adjusted to obtain the best sensitivity (Table S4†). The injection time of 8 s and combination of potentials presented in Fig. 2B were applied to allow the adequate contact with the sample and obtain the highest signal, while not reaching the separation channel in excess. In order to improve the efficiency of the interaction between antibody and analyte or analyte–enzyme conjugated, the flow was stopped. An incubation time of 420 s was considered, since a longer time did not show an appreciable increment of the signal (Table S5†). Elimination of any traces of unbound species was conducted by a reverse flow which flushes them towards the sample reservoir (Fig. 2D).

After the immunological complex was constructed and excess of reagents were eliminated, the modified magnetic beads were strategically moved to the enzymatic reaction zone, thanks to the

aid of a removable magnet. Afterward, the enzymatic reaction related with the analyte concentration in the sample proceeded according to the previous optimized conditions stated above. Under these optimized conditions, the integrated microfluidic electrochemical immunoassay took a total analysis time of about 15 minutes improving the time taken in conventional procedures.

3.3 Microfluidic immunoassay analytical characteristics evaluation

In order to show the capabilities of the system and to predict the ZEA concentrations in unknown samples, a calibration curve was constructed within the interval of 0–1000 μg L^{-1} ZEA concentrations. Fig. 3 shows the calibration plot obtained as well as the analytical signals obtained for the different concentrations assayed (inset). The obtained values were fitted to a four-parameter logistic equation and the limit of detection, calculated as 90% of the maximum signal, was 0.4 μg L^{-1}.

To evaluate the accuracy of the proposed microfluidic immunoassay, maize certified reference material (83 ± 9 μg kg^{-1}) was analysed. After extraction and dilution, 100-fold in PBS–T–BSA 0.1%, five samples were directly measured. It is necessary to remark that the final concentration reached after dilution is under 1 μg L^{-1} (0.83 μg L^{-1}) well below the legislation requirements. Furthermore and in order to diminish the time consuming and laborious procedure of routinely performing a four-parameter logistic calibration curve for sample analysis, a simplified calibration procedure was also proposed. This strategy implies the determination of a just-selected concentration of ZEA standard with calibration purpose, followed by the analysis of the food sample. Comparison of both signals after the adequate definition of the calibration factor allows the determination of the mycotoxin in the sample. Indeed, the calibration factor is calculated as $f_{\text{calibration}} = S_{\text{ZEA standard}} \times [\text{ZEA}]_{\text{standard}}$. Since this calibration factor is a constant for each analysis, ZEA concentration in the sample can be calculated as $[\text{ZEA}]_{\text{sample}} = f_{\text{calibration}}/S_{\text{ZEA sample}}$. This methodology is clearly advantageous because it generates a simplification in the immunoanalysis calibration procedure on microfluidics. The results are presented in Table 1, where it can be observed how the values obtained for ZEA concentrations were found within the uncertainty given by reference material (systematic error less than 2%), yielding, in consequence, recoveries close to 100%. This fact clearly demonstrates the adequate suitability of the proposed microchip immunoassay.

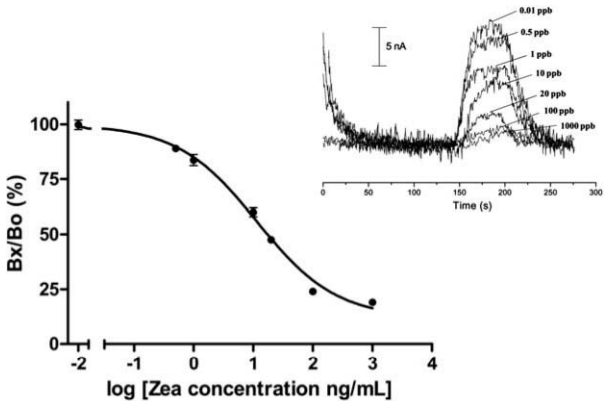

Fig. 3 Zearalenone calibration curve and their related analytical signals (inset) using the competitive electrochemical microfluidic immunoassay.

Table 1 Determination of ZEA in a certified reference[a]

Sample	ZEA concentration/μg kg^{-1}	Recovery (%)
1	82	98
2	86	103
3	80	96
4	83	100
5	88	106
	84 ± 3	101 ± 4

[a] Maize containing ZEA at 83 ± 9 μg kg^{-1}.

The precision of the electrochemical microfluidic immunoassay has carefully been checked with ZEA control solutions at concentrations of 0 (maximum signal), 1 and 20 μg L^{-1}. The intra-assay precision was evaluated with five measurements at the same level of concentration in five runs performed consecutively during the same day. These series of analysis were repeated for five different days to estimate inter-assay precision. The results obtained are summarised in Table 2. While the CV within-assay values obtained are quite acceptable for the different concentration levels, an increment for the CV obtained in different days can be observed. Although these variations observed at all concentrations assayed during different days are not so much unusual in immunoassays, they can be dramatically diminished when a control (maximum signal, 0 μg L^{-1}) is used and data are expressed as a percentage from the control. That means that absolute signal could vary for different days, although the relation between different concentration signals and the maximum signal remains almost constant. In consequence, the use of the control is recommended to improve the analytical performance.

3.4 Analytical strategy for reliable control of permitted levels of zearalenone in infant foodstuff

Because of the good accuracy and precision obtained for the microfluidic immunosensor, analysis of food samples concerned was carried out.

Given that legislation requirements for baby food samples set the maximum amount of ZEA at 20 μg kg^{-1}, and the fact that both solid and liquid samples can contain the mycotoxin, we have established two performance strategies depending on the nature of the sample. In both cases, references values for sample discrimination were established.

Table 2 Precision of microfluidic electrochemical immunoassay

ZEA concentration/μg kg^{-1}	Intra-assay[a]/CV%	Inter-assay[b]/CV%	
0	4	17	
1	9	16	3[c]
20	7	18	3[c]

[a] n = 5, same day. [b] n = 5, different days. [c] Calculated from data expressed as a percentage from the maximum signal control.

Table 3 Determination of permitted levels of ZEA in infant solid foods (powdered)[a]

Maximun signal[b]/nA	Reference signal[c]/nA	Sample signal/nA	ZEA concentration/μg kg^{-1}	Recovery (%)
14.3	12.2	11.7	1.04	104
14.1	11.9	11.9	1.00	100
15.2	12.1	11.2	1.08	108
15.1	12.0	11.8	1.02	102
15.4	12.4	12.3	1.01	101
14.8 ± 0.6	12.1 ± 0.2	11.8 ± 0.4	1.03 ± 0.03	103 ± 3

[a] Spiked at 20 μg kg^{-1} ZEA concentration. After extraction and dilution the final concentration in the sample is 1 μg L^{-1}. [b] Maximun signal obtained for non-spiked ZEA. [c] Reference signal obtained for 1 μg L^{-1} of ZEA standard.

On one hand, solid samples require an extraction procedure and dilution step to obtain the mycotoxin in solution where the influence of organic solvents must be minimised. In this sense, when solid samples are spiked at the concentration level of 20 μg kg^{-1}, treated by extraction and dilution according to the procedure, they reached a final concentration of 1 μg L^{-1}. For this reason, we have used a ZEA standard of 1 μg L^{-1} as the reference value to estimate the presence of the mycotoxin in the solid sample. Samples producing lower or higher currents than that produced by the 1 μg L^{-1} standard could be considered as positive or negative respectively. In this sense, a powdered baby food containing different types of cereals, and previously found to contain an undetectable level of the mycotoxin, was afterwards spiked at the maximum tolerable limit of 20 μg kg^{-1}. After extraction and dilution, analysis of samples was performed using the simplified calibration method stated above. Table 3 lists the results obtained during the control of the maximum permitted level of ZEA for solid samples.

On the other hand, in the case of liquid samples, although matrix interferences due to the presence of mainly proteins or lipids could be observed, determination without pre-treatment can be accomplished. In this sense, as no extraction or dilution process is needed, the current generated by a standard of 20 μg L^{-1} can be directly used as a reference value to assign the positive or negative to the samples. A cereal milkshake, as example of liquid samples was also analysed by the microfluidic immunoassay. Samples were spiked at 20 μg L^{-1} ZEA concentrations and directly analysed without any extraction procedure or dilution step. The results obtained for the analysis of liquid samples are listed in Table 4. Either for solid or liquid samples, the integrated microfluidic immunoassay has demonstrated the suitability for determination of the mycotoxin at the maximum tolerable level permitted for the most restricted regulations. Going beyond and in order to confirm the capability of the method to differentiate those samples considered non-toxic samples (content of the mycotoxin bellow 20 μg kg^{-1}), lower concentrations in solid and liquid samples were studied. In this case, the solid and liquid real samples were spiked at 10 μg L^{-1}. As mentioned previously, the liquid samples were directly assayed and compared with the 20 μg L^{-1} reference standard, while solid samples were extracted and diluted reaching in this case a final concentration value of 0.5 μg L^{-1} and related to the 1 μg L^{-1} reference concentration. In Table S6†, an adequate determination of the mycotoxin for the milkshake liquid sample can be observed, while an overestimation of the concentration was obtained for the diluted solid sample. An explanation of this behaviour can be provided taking into account that 0.5 μg L^{-1} is close to the detection limit of the method and clearly out of the linear range of the calibration curve. Nevertheless, it is worthy to remark that the signal obtained for the diluted sample was higher than for the control one, indicating a lower concentration of the sample. In addition, the comparison with the control of maximum signal (ZEA 0 μg L^{-1}) showed a lower signal for the samples and a percentage near the one corresponding with the limit of detection. These facts clearly point out the goodness of the microfluidic immunosensor method developed.

4. Conclusions

This work has clearly demonstrated that reliable electrochemical immunoassay for the control of levels of ZEA in infant foods has been successfully integrated on microfluidic chips. Based on a simple double-T microchip layout but exploiting a creative strategy where both channels are used as immunological and enzymatic reaction chambers, the usually associated problem of non-specific adsorption of proteins to the wall-channel was avoided. Accuracy of the proposed microfluidic immunoassay was established by the analysis of a certified reference material, while a simplified calibration procedure allowed diminishing time consuming and laborious procedure of performing a four-

Table 4 Determination of permitted levels of ZEA in infant liquid foods (milkshake)[a]

Maximun signal[b]/nA	Reference signal[c]/nA	Sample signal/nA	ZEA concentration/μg L^{-1}	Recovery (%)
17.8	8.3	8.0	20.8	104
20.5	9.4	9.3	20.2	101
19.1	8.0	8.2	19.5	97
17.2	9.2	9.4	19.6	98
17.4	8.5	8.1	20.9	104
18.0 ± 1.0	8.7 ± 0.6	8.6 ± 0.7	20.2 ± 0.6	101 ± 3

[a] Spiked at 20 μg L^{-1} ZEA concentration. [b] Maximum signal obtained for non-spiked ZEA. [c] Reference signal obtained for 20 μg L^{-1} of ZEA standard.

parameter calibration curve. Either solid or liquid food samples were analysed, and those contaminated above the permitted levels of the mycotoxin could be easily discriminated by the developed methodology. These results are inherently very promising since they offer a new and valuable alternative to using microfluidic chips as bio-sensing platforms.

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INTEGRATED ELECTROKINETIC MAGNETIC BEAD-BASED ELECTROCHEMICAL IMMUNOASSAY ON MICROFLUIDIC CHIPS FOR RELIABLE CONTROL OF PERMITTED LEVELS OF ZEARALENONE IN INFANT FOODS

SUPPORTING INFORMATION
(For publication)

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Table S1. Optimization of the volume of modified magnetic beads

Beads volume (μL)	Signal (nA)
0.1	9.0
0.2	11.0
0.5	8.3
1.0	4.5
2.0	2.1

Table S2. Analytical signals (nA) obtained during the optimization of electrokinetic injection of substrate and electrochemical mediator.

Injection time (s)	Injection potential (V)			
	1500	**2000**	**2500**	**3000**
10	0.0	0.0	3.5	7.6
20	0.0	3.5	5.1	13.0
40	5.1	7.8	7.1	10.6
60	6.5	8.7	10.0	N.D.
80	6.9	N.D.	N.D.	N.D.

N.D.: distorted and broad peak.

Table S3. Optimization of electrokinetic flow rate.

Driving potential (V)	Signal (nA)	Migration time (s)
1000	6.5	700
1250	9.3	465
1500	10.7	281
1750	9.4	215

Table S4. Optimisation of the sample/tracer injection time for the immunological interaction[1].

Injection time (s)	Signal (nA)
3	2.0
6	4.1
8	8.4
10	8.0

[1] **Incubation time was fixed to 420 s.**

Table S5. Optimization of the incubation time for the immunological interaction[1].

Incubation time (s)	Signal (nA)
60	N.D.
300	8.4
360	14.1
420	15.0

[1]Injection time was fixed to 8 s.

Table S6. Determination of ZEA under permitted levels in infant liquid (milkshake) and solid foods (powdered) [1]

Maximum signal[2] (nA)	Reference signal[3] (nA)	Liquid sample signal (nA)	ZEA concentration (μg L^{-1})	Recovery (%)
14.1	6.5	12.0	10.8	108
15.9	6.7	12.5	10.7	107
17.7	6.1	11.2	10.9	109
15.9±1.8	6.4±0.3	11.9±0.6	10.8±0.1	108±1
Maximum signal[2] (nA)	**Reference signal[4](nA)**	**Solid sample signal (nA)**	**ZEA concentration (μg L^{-1})**	**Recovery (%)**
19.6	16.1	18.2	0.88	176
21.1	16.8	18.5	0.90	180
22. 4	16.4	18.3	0.89	178
21.0±1.0	16.4±0.4	18.4±0.2	0.89±0.01	178±2

[1] Spiked at 10 μg L^{-1} (For the solid sample, the final concentration was 0.5 μg L^{-1}). [2] Maximun signal obtained for non-spiked ZEA. [3] Reference signal obtained for 20 μg L^{-1} of ZEA standard for milkshake (liquid sample). [4] Reference signal obtained for 1 μg L^{-1} of ZEA standard for powdered (solid sample).

III.6 EXPOSICIÓN Y DISCUSIÓN CONJUNTA

En esta sección se comentan de forma crítica y comparada el conjunto de todos los resultados obtenidos en este trabajo de investigación, poniendo de manifiesto las principales fuerzas y debilidades de los mismos.

III.6 EXPOSICIÓN Y DISCUSIÓN CONJUNTA DE LOS RESULTADOS MÁS RELEVANTES

Aunque cada una de las estrategias analíticas desarrolladas en esta Tesis Doctoral se han recogido en las diferentes secciones que constituyen el capítulo de Resultados y Discusión en forma de las respectivas publicaciones obtenidas, se ha creído conveniente llevar a cabo una discusión conjunta sobre la evolución natural de todas las aproximaciones analíticas desarrolladas, que tienen todas en común el estar basadas en inmunoanálisis electroquímico para la detección y control de la micotoxina zearalenona (ZEA) en muestras de interés agroalimentario.

Como se ha indicado en la introducción de esta memoria, uno de los campos más importantes en el análisis de alimentos, es la determinación de niveles extremadamente bajos de toxinas y micotoxinas. Dentro de las micotoxinas, la ZEA ha sido objeto de estudio por parte de la comunidad científica debido a su posible presencia en un gran número de alimentos básicos de la dieta y a los efectos adversos que origina sobre la salud humana y animal.

En base a su toxicidad, ha resultado preciso establecer unos límites para proteger a la población de los efectos nocivos que provocan los alimentos contaminados por esta micotoxina. Sin embargo, no existe un límite máximo internacional armonizado, por lo que la cantidad máxima permitida de ZEA, tanto en alimentos destinados al consumo humano como para animales, varía de unos países a otros siendo 20 μg Kg^{-1} (ppb) el límite más restrictivo.

A su vez, muchas micotoxinas como ZEA no están estudiadas en profundidad, y debido a los requerimientos cada vez más exigentes de la legislación (sobre todo cuando se trata de alimentos infantiles), es probable que en los próximos años aparezcan nuevos límites, cada vez más restrictivos con objeto de mejorar su regulación y control en alimentos. Por tanto, ha surgido la necesidad de desarrollar métodos cada vez más sensibles y específicos, que

permitan la rápida detección de ZEA en alimentos, muy por debajo de los actuales límites máximos permitidos.

En la actualidad, los métodos inmunoanalíticos han llegado a ser la metodología de elección para la determinación de micotoxinas en general y de ZEA en particular debido a las excelentes características que presentan estos métodos, tales como: (i) su elevada especificidad y sensibilidad, y (ii) su relativa rapidez, sencillez y bajo coste frente a técnicas más sofisticadas (HPLC, GC, MS).

Por otra parte, la detección electroquímica, en términos generales es una herramienta muy valiosa debido a su elevada sensibilidad y bajo coste. En el campo del inmunoanálisis constituye una de las herramientas básicas debido al buen comportamiento de reacciones enzimáticas acopladas a reacciones de transferencia de carga que producen sensibilidades extraordinarias. Sin embargo, su principal protagonismo lo alcanza en el ámbito más contemporáneo de la miniaturización y microfluídica analítica donde constituye la más importante alternativa a la detección óptica debido a su miniaturización inherente y a su compatibilidad con las tecnologías de serigrafiado, de microfabricación y con las nuevas nanotecnologías.

En este sentido y siguiendo la evolución de los objetivos planteados en este trabajo de investigación y que se han recogido en el capítulo II de esta memoria, se han desarrollado nuevas estrategias analíticas basadas en inmunoensayo electroquímico para asegurar la detección y el control de la micotoxina ZEA en muestras de cereales. Los resultados obtenidos a lo largo de esta tesis y recogidos precedentemente en las secciones III.1; III.2; III·4 y III.5 en forma de las publicaciones científicas obtenidas, se expondrán y discutirán comparativamente a continuación.

Desarrollo de un ELISA electroquímico en placa para la detección y control de ZEA en alimentos infantiles. (*"Electrochemical immunoassay using magnetic beads for the determination of zearalenone in baby food: An anticipated analytical tool for food safety")*

Si se vigilan los objetivos fijados en este trabajo de investigación, es preciso indicar que el punto de partida lo constituye el desarrollo de una metodología ELISA en formato convencional en placa, aunque con detección electroquímica, y a partir de la misma, se evoluciona de forma conceptual y natural hacia dos estrategias miniaturizadas con diferente abordaje: el desarrollo de un inmunosensor y la integración total del ELISA en una plataforma microfluídica. Con independencia de la estrategia desarrollada, el principio inmunoanalítico electroquímico así como la estrategia de llevarlo a cabo soportado sobre partículas magnéticas, es común.

Con tal motivo, en una primera gran etapa previa, se estudiaron todos los aspectos básicos relacionados con esta arquitectura común que constituyen el sustrato del trabajo primero que conforma esta Tesis. En efecto, el objetivo de este trabajo fue el diseño y desarrollo de un método inmunoanalítico electroquímico ELISA soportado en partículas magnéticas para asegurar la detección y el control de la micotoxina ZEA en muestras que contuviesen cereales.

Esta propuesta tuvo como finalidad desarrollar una ruta alternativa y competitiva a las ya existentes, las cuales emplean instrumentación analítica cara y sofisticada, que a su vez pueda servir de punto de partida para la exploración de nuevas estrategias dentro del escenario más contemporáneo de la Química Analítica.

En primer lugar, se evaluaron las características tecnológicas de los reactivos especializados adquiridos comercialmente (anticuerpo para ZEA y el conjugado enzimático) mediante el método ELISA tradicional en placa con detección espectrofotométrica. A continuación, y una vez valorados dichos reactivos inmunológicos, se diseñó y optimizó un ELISA utilizando partículas magnéticas como soporte de inmovilización y detección electroquímica sobre electrodos serigrafiados de carbono (CSPE).

Como se ha indicado anteriormente, en este trabajo se han utilizado partículas magnéticas recubiertas de proteína G como soporte de inmovilización, que permiten por una parte, conseguir la inmovilización orientada de los anticuerpos (debido a la interacción de la proteína G con la región Fc del anticuerpo) y por otra, hacer uso de las características aportadas por la partículas magnéticas (fácil manipulación mediante un campo magnético, mejora en las cinéticas de reacción), mejorando así la sensibilidad y reproducibilidad del ensayo. La **Figura 17** ilustra estas dos ventajas.

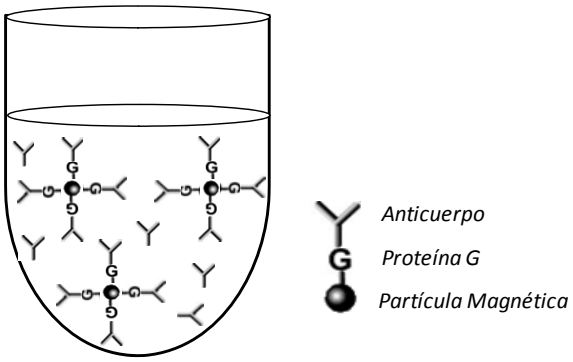


Figura 17. Unión esquemática de la región Fc del anticuerpo con la proteína G.

Por su parte, el inmunoensayo se desarrolla en base a un esquema competitivo directo, en el que la micotoxina ZEA y el conjugado enzimático (ZEA-HRP) compiten por los sitios de unión del anticuerpo específico para ZEA. De tal modo, que la cantidad de conjugado enzimático retenido por su interacción con el anticuerpo será inversamente proporcional a la concentración de ZEA en la muestra (o patrón). Una vez que ha tenido lugar el reconocimiento molecular (reacción antígeno-anticuerpo), la determinación de ZEA se basa en la detección amperométrica de la actividad de la enzima (HRP) que ha quedado retenida por la interacción del conjugado enzimático con el anticuerpo. La peroxidasa (HRP) en presencia de peróxido de hidrógeno cataliza la oxidación de hidroquinona (mediador electroquímico) a benzoquinona, cuya reducción electroquímica en la superficie electródica es detectada. La **Figura 18** ilustra el esquema del inmunoensayo llevado a cabo.

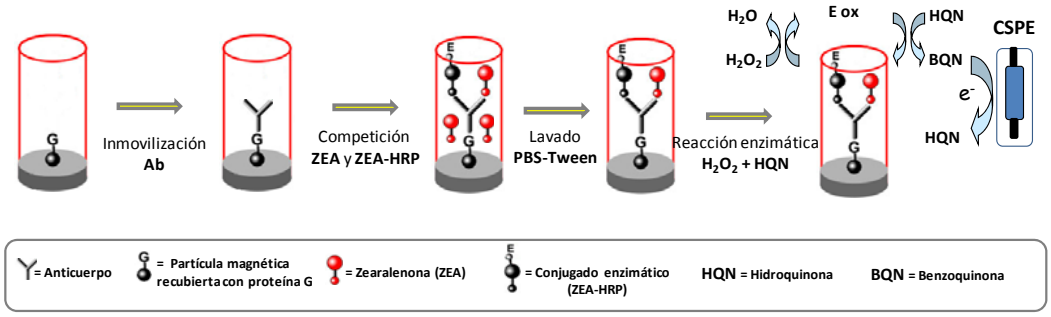


Figura 18. Representación esquemática del ELISA en placa con detección electroquímica para la detección de ZEA.

Este diseño correspondiente al desarrollo y optimización de un ELISA electroquímico en placa de microtitulación presenta dos partes perfectamente definidas. En primer lugar, se ha llevado a cabo la optimización de las variables que configuran la arquitectura final del inmunoensayo propuesto y en segundo lugar, la evaluación del método bajo el marco de la legislación vigente tomando como referencia las dos propiedades analíticas que hacen válida a dicha estrategia para el control de seguridad en alimentos: el límite de detección (LOD) que nos asegure la detección a los niveles de concentración exigidos por la legislación y la exactitud que nos permita asegurarnos sobre el nivel de micotoxina medido, en su caso. Adicionalmente, es preciso reflexionar sobre el hecho de que la sociedad del bienestar demanda cada vez más una mayor seguridad alimentaria y se prevé, al igual que ya ha sucedido con otras problemáticas, una legislación cada vez más estricta, especialmente para alimentos infantiles, por lo que el LOD es una propiedad analítica muy importante en este ámbito.

En cuanto a la optimización de los distintos parámetros que configuran la metodología utilizada, en el epígrafe 3.1 de la página 67 y correspondiente al artículo 1 se encuentra una descripción detallada del estudio de los mismos junto a los resultados obtenidos. Es preciso resaltar que la elección adecuada de estas variables repercute de forma directa en las características analíticas de la metodología desarrollada. En este sentido, la concentración del anticuerpo específico y el conjugado enzimático, resulta un aspecto clave que afecta a la sensibilidad de los ensayos competitivos. De forma habitual, cuanto menor es la concentración de dichos reactivos mejor es su LOD, aunque deben estar en concentración

suficiente para producir una sensibilidad adecuada. Además, en este caso, la cantidad de anticuerpo específico inmovilizado vendrá limitada por la cantidad de partículas magnéticas modificadas con proteína G que constituyen la base de la arquitectura formada. Del mismo modo, las concentraciones de substrato enzimático y mediador electroquímico deben ser suficientemente elevadas para no constituirse como un factor limitante, y permitir que la respuesta generada venga condicionada por la cantidad de enzima retenida en la reacción de afinidad. Atendiendo a los principios comentados y en base a los resultados obtenidos, se propusieron las condiciones y valores optimizados para el desarrollo de la metodología propuesta que a modo de resumen se muestran en la siguiente tabla.

Tabla 2. Resumen de los parámetros optimizados.

Partículas magnéticas-proteína G	Concentración anticuerpo	Tiempo incubación (Ab)	Factor dilución (Z-HRP)	Tiempo incubación (Z-HRP)	Concentración substratos enzimáticos	Tiempo incubación S.E.
2 µL	5×10^{-3} mg mL^{-1}	30 min	1:200	30 min	H_2O_2: 600 µM HQN: 800 µM	25 min

Es preciso subrayar, que de forma previa fue necesario un estudio riguroso de la posible adsorción inespecífica del conjugado enzimático tanto a las propias partículas magnéticas como a las paredes del vial, lo que podría conllevar una señal residual de fondo y perjudicar la sensibilidad del método. Distintos experimentos realizados en ausencia del anticuerpo específico, así como con una concentración elevada de la propia micotoxina, produjeron una señal del orden del 60% respecto de la máxima señal correspondiente a la propia interacción anticuerpo/conjugado enzimático. Incluso, la sola adición del conjugado sobre el vial utilizado para el ensayo (bloqueado con PBST-BSA-1%) producía una señal del 38 %. Esto nos llevó a la necesidad de sustituir los viales de vidrio donde tenía lugar la reacción inmunológica por pocillos de placas de ELISA, que tras la correspondiente etapa de bloqueo (PBST-BSA-1%) redujo la adsorción inespecífica a valores en torno al 19%.

Con respecto a la evaluación del método, es preciso indicar que, el *alma mater* de los objetivos de esta Tesis lo constituye el desarrollo y evaluación de nuevas metodologías de inmunoensayo y no la aplicación profusa de las mismas a un conjunto de muestras agroalimentarias. Con tal motivo, y con independencia de la aproximación analítica desarrollada, se han elegido para la validación, por una parte, un material de referencia certificado (MRC) de ZEA en maíz cuya concentración es de 83±9 μg Kg^{-1} y por otra, dos muestras analíticas entendidas como representativas de la problemática, correspondientes a alimentos infantiles con matrices de diversa complejidad conteniendo cereales y de inequívoco interés. Estas muestras objeto de análisis, corresponden a una matriz líquida representada por un batido lácteo con cereales y a una matriz sólida, que consiste en una papilla de cereales en polvo, ambas destinadas al consumo infantil. Las muestras escogidas con carácter representativo, se analizan de forma sistemática utilizando las distintas estrategias desarrolladas con objeto de comprobar la viabilidad de las metodologías propuestas.

Los resultados obtenidos han demostrado que el método propuesto es un método válido y altamente competitivo frente a los ya existentes, debido a que el límite de detección encontrado (0.011 μg L^{-1}) está muy por debajo de los niveles permitidos por la legislación vigente (20 μg Kg^{-1}) y es del mismo orden que el mejor de los encontrados en la bibliografía[95]. Así mismo, la reproducibilidad intra-laboratorio evaluada a partir de cuatro curvas de calibrado realizadas en distintos días y en un intervalo de concentraciones de 0-1000 μg L^{-1} dio lugar a un valor medio de desviación estándar relativa de 7.8%. Este dato demuestra, claramente, la buena fiabilidad del método en términos de precisión.

En cuanto a la exactitud del método propuesto, se ha evaluado mediante el análisis de un MRC de ZEA en maíz (83±9 μg Kg^{-1}). Los resultados obtenidos presentan unos porcentajes de recuperación que oscilaron entre el 95 y el 108%, situando los valores de concentración de ZEA determinada dentro del intervalo de confianza correspondiente al MRC. Estos valores que pueden verse en la Tabla 1, de la página 69, correspondiente al artículo 1, han confirmado la excelente exactitud del método desarrollado. Es importante remarcar que el

tratamiento de las muestras sólidas correspondientes tanto al material de referencia certificado, como la papilla de cereales en polvo, han requerido de un paso previo de extracción. Este paso de extracción se ensayó utilizando dos combinaciones distintas de disolventes, no habiéndose encontrado diferencias significativas tal y como se muestra en la Tabla 1 del artículo 1 (página 69). Tras la etapa de extracción, y previo al análisis, es preciso un paso de dilución con objeto de evitar la acción de los disolventes en la interacción antígeno-anticuerpo.

Una vez demostrada la exactitud del método y la posibilidad de llevar a cabo su cuantificación en los niveles de concentración requeridos, se ha llevado a cabo la determinación de ZEA en las muestras reales escogidas y comentadas anteriormente.

Una de las grandes ventajas de la metodología inmunoanalítica desarrollada, es su posible aplicación directa sobre muestras de naturaleza líquida, gracias a la selectividad y sensibilidad características de la misma. En este sentido, se ha procedido a la determinación directa de ZEA en un batido de cereales, sin necesidad de realizar pasos de extracción ni dilución de la muestra. Para ello y en orden a evaluar posibles efectos de matriz, se realizó una curva de calibrado directamente sobre la matriz sin diluir y se comparó con la curva de calibrado realizada en buffer. Los valores obtenidos tanto para el EC_{50} (1.5 μg L^{-1} respecto a 0.079 μg L^{-1} en buffer) como para el límite de detección (0.12 μg L^{-1} respecto a 0.011 μg L^{-1} en buffer) fueron ligeramente superiores, indicando la presencia de cierto efecto de matriz. A continuación y una vez demostrada la ausencia de la micotoxina en la muestra, se procedió a fortificar la matriz líquida a tres niveles de concentración de ZEA (1, 10, 100 μg L^{-1}). Utilizando una curva de calibrado realizada en la propia matriz, se obtuvieron los resultados que se muestran en la Tabla 3 (página 70, artículo 1) donde se observa un intervalo de recuperaciones comprendido entre 96 y 104 %, demostrando así la fiabilidad del método.

Respecto al análisis de recuperación en una papilla de cereales, previamente ha sido fortificada a dos niveles de concentración: 100 y 20 μg Kg^{-1}, simulando en este último caso

una muestra sólida cuya concentración estaría en el límite más restrictivo permitido por la legislación. Tras la etapa de extracción y dilución de la muestra, las concentraciones finales de las muestras sometidas al análisis son 0.25 y 0.05 µg L^{-1}, respectivamente. Los resultados obtenidos también mostraron una buena concordancia entre la concentración de ZEA añadida y la encontrada, con unos porcentajes de recuperación del 108 y 140 % (ver Tabla 2, página 69, artículo 1). Estos valores, denotan sin duda, la fiabilidad del método incluso a concentraciones muy bajas.

En consecuencia, se puede deducir que, la estrategia analítica propuesta basada en inmunoensayo electroquímico soportado en partículas magnéticas, ha demostrado ser una herramienta válida para la detección sensible de ZEA en alimentos infantiles; convirtiéndose a su vez, en una herramienta analítica que se anticipa a futuros retos dentro del ámbito de la seguridad alimentaria al haberse obtenido un LOD muy por debajo de los límites admitidos por la legislación vigente.

A pesar de los excelentes resultados obtenidos mediante la aplicación de la metodología anteriormente desarrollada, hay que hacer constar la existencia de ciertos inconvenientes que pueden ser mejorados. Fundamentalmente los debidos a los largos tiempos de análisis empleados (aproximadamente 90 min), y la tediosa calibración metodológica requerida. Por todo ello, se ha procedido a la búsqueda de alternativas miniaturizadas que mejoren algunos de estos aspectos analíticos dentro de ese escenario más contemporáneo de la Química Analítica, ya comentado con anterioridad.

En este sentido, una de las tendencias más dominantes en Química Analítica es aquella que persigue el desarrollo de sistemas miniaturizados, automáticos y simplificados, que integren múltiples procesos en un único dispositivo, sean portátiles y por ello permitan la descentralización de los laboratorios, reduzcan la intervención humana (automatización) y permitan, en general, la simplificación de los procesos. Dos grandes estrategias se han ido desarrollando en Química Analítica bajo estas coordenadas: la tecnología de sensores y biosensores, así como el desarrollo de sistemas de análisis total microfluídicos basados en la

tecnología del "lab-on-a-chip". En esta Tesis, se han abordado ambas estrategias partiendo de la base analítica desarrollada en la etapa anterior. Por una parte, se ha desarrollado un inmunosensor electroquímico y, por otra, un sistema "lab-on-a-chip" integrando el inmunoanálisis electroquímico; ambas para la detección y control de ZEA en alimentos infantiles.

Desarrollo de un inmunosensor electroquímico, con calibración simplificada y haciendo uso de CSPE desechables, para la determinación de ZEA en alimentos infantiles. *"Simplified calibration and analysis on screen-printed disposable platforms for electrochemical magnetic bead-based immunosensig of zearalenone in baby food samples"*

La tecnología de biosensores ha experimentado un notable avance en los últimos años, como se refleja en el gran número de artículos científicos que genera. Dentro de ellos, los inmunosensores se han convertido en una herramienta analítica ideal y en una alternativa a los métodos inmunoanalíticos clásicos, debido fundamentalmente a sus características que nos permiten obtener información rápida, selectiva, sensible, económica y a tiempo real. Entre ellos, los inmunosensores amperométricos son los que han mostrado un mayor desarrollo. En este sentido, el siguiente objetivo de este trabajo de Tesis se centró en el desarrollo de un inmunosensor, de manera que la arquitectura correspondiente al inmunocomplejo formado se encontrase en íntimo contacto con un transductor electroquímico.

Por ello, el objetivo correspondiente a esta etapa metodológica fue el desarrollo de un inmunosensor electroquímico soportado sobre partículas magnéticas y llevado a cabo directamente sobre la superficie de electrodos desechables serigrafiados, para la detección de ZEA en muestras alimentarias infantiles. Esta propuesta tuvo como finalidad desarrollar una herramienta analítica sencilla y desechable para análisis rápidos. Para ello la tecnología serigrafiada ofrece unas ventajas inmejorables.

Del mismo modo que en el desarrollo de la estrategia anterior, en una primera etapa se optimizaron las variables que configuran la arquitectura final del inmunosensor

electroquímico propuesto, para posteriormente evaluar sus propiedades analíticas. En este sentido, y tras prestar especial atención al LOD y exactitud obtenidos, se analizaron las muestras seleccionadas para comprobar la validez de la estrategia propuesta.

El procedimiento analítico se llevó a cabo de forma similar al correspondiente ELISA desarrollado en la estrategia 1. Por tanto, la reacción inmunológica está basada en un ensayo competitivo en el cual las partículas magnéticas son utilizadas como soporte de inmovilización y la enzima peroxidasa (HRP) se utiliza como marcador enzimático. La diferencia fundamental estriba en la etapa de detección. En este caso, una vez finalizada la reacción de bioreconocimiento, las partículas magnéticas transportando los correspondientes inmunocomplejos, son confinadas, gracias a la ayuda de un imán, en la superficie de un electrodo serigrafiado con configuración de tres electrodos. Esta configuración permite realizar la detección en la superficie del electrodo en forma de gota, donde se incluyen las partículas magnéticas modificadas, sin la necesidad de una celda adicional. En la **Figura 19** se muestra un esquema de la arquitectura propuesta para el inmunosensor. La detección electroquímica se realizó mediante la adición de 10 μL de una disolución que contenía el sustrato enzimático (H_2O_2) y mediador electroquímico (Hidroquinona) sobre la gota de disolución reguladora depositada en la superficie del electrodo, de modo que se cubra el sistema de tres electrodos del CSPE. A continuación, y tras un tiempo de incubación de 5 min, la respuesta del sistema se obtiene por voltametria diferencial de pulso.

La estrategia de llevar a cabo el desarrollo de esta metodología en dos etapas que, además tienen lugar en diferentes localizaciones conlleva una serie de ventajas. Por un lado, la etapa de bioreconocimiento tiene lugar en disolución, lo que permite aprovechar los beneficios de usar partículas magnéticas como una mayor área superficial, mayor probabilidad de que las diferentes inmunoespecies entren en contacto, así como la facilidad de su manipulación. Por otro lado, confinar las partículas magnéticas en la superficie del electrodo para llevar a cabo la etapa de detección evitando la pasivación (recubrimiento) del electrodo por la deposición de los anticuerpos específicos, mejorando la transferencia de electrones, a la vez

que reduce la adsorción inespecífica, debido al hecho de que la reacción de afinidad no se realiza en la superficie del electrodo.

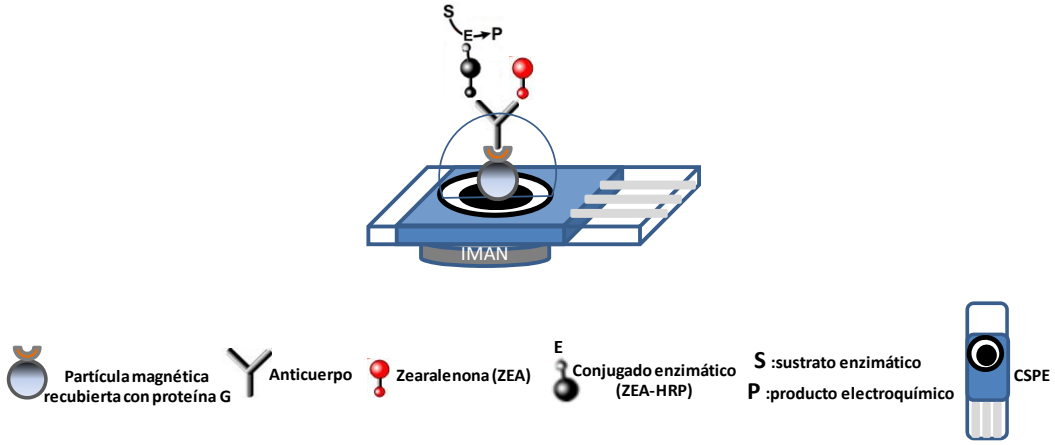


Figura 19. Representación esquemática del inmunosensor electroquímico para la detección de ZEA.

Las condiciones de ensayo, utilizadas en la etapa correspondiente a la reacción de bioreconocimiento, han sido optimizadas en la estrategia anterior y resumidas en la **Tabla 2**. Sin embargo, los parámetros correspondientes a la detección electroquímica hubieron de ser estudiados de nuevo. En este sentido, las concentraciones de substrato y mediador electroquímico fueron re-optimizadas y, asimismo, fue necesario comprobar el posible efecto del campo magnético empleado para el confinamiento de las partículas sobre la superficie electródica sobre el comportamiento electroquímico de la hidroquinona. En cuanto a este último aspecto, los correspondientes ciclovoltamperogramas realizados a la benzoquinona (producto redox obtenido en la reacción enzimática) en ausencia y presencia de las partículas magnéticas, demuestran claramente que no afectan a su respuesta electroquímica (información suplementaria artículo 2, página 79). Por otro lado, y teniendo en cuenta la configuración en gota, se recurrió a la voltametria diferencial de pulsos para obtener una proporcionalidad entre la señal obtenida y la concentración de la micotoxina.

Con respecto a los resultados más relevantes obtenidos para el inmunosensor desarrollado, resulta preciso resaltar como a pesar de las modificaciones realizadas en la etapa de

detección, el valor obtenido para el límite de detección se ha mantenido en el mismo orden de magnitud (0.007 μg L^{-1}). Este valor se encuentra también muy por debajo de los niveles permitidos por la legislación vigente (20 μg Kg^{-1}). Asimismo, el tiempo total de análisis se ve reducido en 20 min aproximadamente.

En referencia a la exactitud del inmunosensor, y siguiendo las pautas de validación planteadas inicialmente, ha sido evaluada mediante la utilización del citado MRC de ZEA en maíz. Los resultados obtenidos resultaron ser excelentes debido a que en todos los casos, el valor correspondiente a los niveles encontrados de ZEA empleando el inmunosensor, estaban comprendidos en el intervalo de confianza del MRC, con un intervalo de recuperaciones del 101 al 111 %. Estos datos avalan la adecuada exactitud del inmunosensor desarrollado.

Una vez demostrado que la nueva configuración de detección utilizada en el immunosensor desarrollado garantizaba su aplicabilidad en cuanto al LOD y la exactitud, se llevó a cabo la medida de ZEA en los dos tipos de muestras reales seleccionadas: una matriz sólida (papilla de cereales) y una matriz líquida (batido de cereales). Las recuperaciones cuantitativas obtenidas para este tipo de muestras resultaron ser del 101±4% para la muestra sólida y del 98±1% para la muestra líquida, tal y como se refleja en las Tablas 2 y 3 de la página 76 del artículo 2.

Sin embargo, una de las principales aportaciones llevadas a cabo en este trabajo de investigación ha sido la propuesta de un protocolo de calibración simplificada empleando un inmunosensor. En este sentido, se pretende simplificar el tedioso protocolo de calibración que exige el trazado completo de la función logística log-log*it* utilizada de forma habitual. Esta estrategia implica, tras la obtención y estudio previo de la curva de calibrado, la calibración metodológica empleando una sola concentración de ZEA próxima al IC_{50}. A continuación, se realiza la medida correspondiente al análisis de la muestra alimentaria utilizando el mismo electrodo para la medida de ambas señales. La comparación de ambas señales permite llevar a cabo la determinación de ZEA en la muestra de forma muy sencilla.

Esta estrategia exige una definición adecuada del factor de calibración ($F_{cal.}$) como el producto obtenido por la señal del patrón y su concentración debido a que es un método indirecto: $F_{cal} = S_{ZEA}. C_{ZEA}$. Este factor nos permite calcular la concentración de ZEA en la muestra ($C_{ZEA\ Muestra} = F_{cal} / S_{muestra}$).

Con el fin de demostrar la validez de la estrategia de la calibración simplificada, se ha llevado a cabo la determinación de ZEA en diez muestras independientes del MRC utilizando un electrodo diferente cada vez. Como se puede observar en los resultados mostrados en la Tabla 4 de la página 77 del artículo 2, en todos los casos, los valores obtenidos estaban incluidos en el intervalo de confianza del MRC, (con un valor medio de 80±5 μg Kg^{-1} respecto al valor de referencia dado para el MRC de 83±9 μg Kg^{-1}), demostrando así la viabilidad de la estrategia propuesta. Es preciso resaltar el hecho de cómo el empleo de una calibración previa a cada análisis, permite evitar la variación obtenida en la señal analítica debida a la baja reproducibilidad de los electrodos empleados, arrojando en todos los casos resultados de alta exactitud. Esta metodología es claramente ventajosa, ya que simplifica el procedimiento de calibración y en consecuencia, del análisis total. Adicionalmente, una ventaja muy atractiva de esta propuesta de calibración, es que si se conoce aproximadamente el valor de ZEA en la muestra, el contenido de ZEA en el patrón utilizado para la calibración puede ser fijado con la misma concentración que se espera, aumentando de esta manera la precisión del análisis.

En consecuencia, el inmunosensor desarrollado consigue reducir el tiempo total del análisis y simplificar el procedimiento de análisis de muestras mediante la calibración simplificada. Además los resultados obtenidos muestran su fiabilidad en términos de precisión, exactitud y LOD.

Sistemas microfluídicos con detección electroquímica para la integración de métodos inmunoanalíticos y determinación de ZEA en muestras de alimentos

La miniaturización de los sistemas analíticos microfluídicos, tal y como se ha comentado en el apartado III.3, implica cambios en las fuerzas y procesos que intervienen en dichos

sistemas, debido a la reducción de la escala, lo que aporta una serie de ventajas únicas, tales como: (i) la gran relación superficie/volumen de estos sistemas permitiéndose rápidas cinéticas de reacción, (ii) la posibilidad de llevar a cabo muchos ensayos de forma simultánea (*multiplexing),* (iii) la reducción del consumo de muestras y/o reactivos y la generación de desechos y, (iv) un mejor control de los fluidos que permite una mayor regulación sobre las operaciones analíticas necesarias para llevar a cabo un análisis total. Asimismo, la miniaturización total de los sistemas analíticos proporciona múltiples alternativas en el análisis *in situ* debido a la inherente portabilidad de estos dispositivos. Por ello, como continuación natural, un gran objetivo planteado en el desarrollo de esta Tesis, fue estudiar las posibilidades de integración del método ELISA soportado en partículas magnéticas en un sistema microfluídico, con el fin de simplificar y automatizar las etapas del inmunoensayo propuesto. Esta integración se llevó a cabo en dos etapas metodológicas claramente diferenciadas: en la primera de ellas, se exploró la posibilidad de emplear el sistema microfluídico para monitorizar electroquímicamente la ZEA correspondiente al ELISA realizado en placa, y por otra, estudiar las posibilidades de llevar a cabo la integración total del inmunoensayo electroquímico.

Monitorización electroquímica "*on-chip*" de un método ELISA realizado en placa (*off-chip*) para la determinación de ZEA en alimentos infantiles *("Electrochemical microfluidic chips coupled to magnetic bead-based ELISA to control allowable levels of zearalenone in baby foods using simplified calibration").*

En aras de obtener un verdadero "lab-on-a-chip" para el inmunoanálisis que nos ocupa, en un primer nivel de integración, la formación del inmunocomplejo y la reacción enzimática tuvieron lugar "off-chip" (en pocillos individuales de una placa de microtitulación) siguiendo el protocolo optimizado en el desarrollo de la primera estrategia (ELISA en placa con detección electroquímica, artículo 1) y resumido en la Tabla 2. A continuación, el producto enzimático generado (mediador oxidado), fue inyectado electrocinéticamente en los canales de un microchip de vidrio de cruz sencilla, para su monitorización electroquímica "on-chip".

Teniendo en cuenta, por tanto, que el movimiento de fluidos dentro del microchip es de tipo electrocinético y que la detección se lleva a cabo en el propio dispositivo microfluídico, fue necesaria la optimización de las condiciones y parámetros más adecuados para dicha detección. En este sentido, y dado que la configuración de detección empleada tenía un formato "end-channel", el campo eléctrico de arrastre puede afectar al potencial de detección aplicado en el electrodo de trabajo, exigiendo este hecho, una optimización tanto de las condiciones electrocinéticas como de detección. Con tal motivo, se trazaron de nuevo las condiciones electrocinéticas optimizadas (potencial de arrastre de 2000 V y tiempo de inyección de 7 s), así como las curvas hidrodinámicas que se muestran en la Figura 3 (página 134 del artículo 3) y donde se puede deducir que el potencial de detección óptimo correspondió a +0.1V.

En base al diseño del microchip de cruz sencilla utilizado y con fines de conseguir una simplificación metodológica; se propuso, asimismo, la posibilidad de emplear los dos reservorios de inyección con fines de poder integrar la calibración metodológica y el inmunoanálisis propiamente dicho, para la determinación de ZEA. Con tal motivo, se hizo necesaria la adecuada definición de los reservorios del microchip: reservorio de calibración (CR), donde se deposita una concentración perfectamente conocida de patrón de ZEA, reservorio de muestra (RS), donde se depositan las muestras para su análisis y el reservorio de arrastre (RB), donde se deposita la disolución de tampón PBS, que se utiliza como medio electrolítico de arrastre. Esta estrategia se ilustra en la **Figura 20** y ha sido previamente propuesta por nuestro grupo de investigación[113].

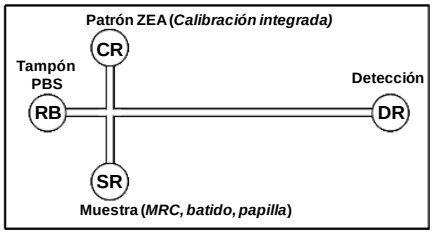


Figura 20. Diseño del microchip de "cruz sencilla" y estrategia para llevar a cabo la calibración (empleando ZEA patrón) y el análisis de las muestras (MRC, batido o papilla) de manera secuencial.

Siguiendo esta estrategia y tras comprobar la adecuada correlación entre distintas concentraciones de ZEA y las señales obtenidas, se procedió a demostrar su exactitud a través del análisis del Material de Referencia Certificado, así como su aplicabilidad en el análisis de muestras reales de interés alimentario ya utilizadas con anterioridad.

La Figura 4 (página 135, artículo 3) muestra los ciclos de señales analíticas correspondientes a las etapas de calibración y análisis empleando el MRC. El cálculo de la concentración de ZEA en el MRC, gracias a la calibración integrada, se lleva a cabo por comparación de las señales obtenidas para el mismo, con aquellas obtenidas para el patrón de ZEA que estratégicamente posee el mismo nivel teórico de concentración que el MRC. En todos los casos los valores obtenidos por el método propuesto están incluidos en el intervalo de confianza del material de referencia, pudiéndose inferir la buena exactitud de nuestro método (ver Tabla 1, página 135, artículo 3).

En el caso de las muestras reales propuestas, se llevó a cabo el correspondiente análisis para evaluar la ausencia de la micotoxina ZEA y por otra parte, el inmunoanálisis para evaluar su presencia en muestras fortificadas a diferentes niveles de concentración, incluyendo tanto la más restrictiva de la legislación como a niveles inferiores.

Tal y como se ha referido con anterioridad, en las muestras líquidas no es necesaria la etapa de extracción, pudiéndose determinar la micotoxina a concentraciones inferiores, limitadas únicamente por el límite de detección del método. De este modo, el batido se fortifica directamente con 0.83 ppb de ZEA, valor muy por debajo de las restricciones actuales. Con objeto de llevar a cabo la calibración integrada con un solo punto de concentración, el reservorio de calibración del chip contiene una disolución patrón de ZEA igual a 0.83 ppb. Los resultados obtenidos muestran un intervalo de recuperaciones comprendido entre 101±7 y 95±4% para las muestras sin fortificar (comprobación de la ausencia de ZEA) y fortificadas, respectivamente.

En el caso de la papilla de cereales, se procede a fortificar la muestra directamente con 20 ppb, teniendo en cuenta que tras la etapa de extracción, el sobrenadante se diluye hasta una

concentración final de 0.83 ppb. Procediendo de la misma forma que en los casos anteriores, se lleva a cabo la calibración integrada con un solo punto de concentración. Los resultados obtenidos muestran un intervalo de recuperaciones comprendido entre (104±3)% y (92±2)% para las muestras sin fortificar y fortificadas, respectivamente.

Adicionalmente, esta estrategia microfluídica presenta importantes ventajas. En primer lugar, debido a que mediante la inyección electrocinética es posible manipular los fluidos y modificar los volúmenes inyectados en el chip, se puede llevar a cabo una reducción del tiempo de incubación del substrato enzimático con el conjugado (de 25 minutos a 15 minutos), si se aumenta el tiempo de inyección (de 7s a 13s) y por tanto se aumenta la cantidad de producto enzimático inyectada (ver Tabla 3, página 137, artículo 3). Por otro lado, con el fin de lograr un acoplamiento robusto del método ELISA y el chip microfluídico, se ha explorado la posibilidad de llevar a cabo la inyección del producto enzimático directamente desde los pocillos ELISA, que se utilizan como reservorios externos del chip. Este nuevo formato muestra unos buenos resultados y una buena reproducibilidad, que indica la flexibilidad y posibilidad de realizar análisis en paralelo, ya que ambos formatos demuestran ser igualmente adecuados (ver Tabla 3, pág. 137, artículo 3).

Los resultados obtenidos pueden considerarse excelentes, demostrando la aplicabilidad del método para la determinación de ZEA en muestras representativas alimentarias de elevado interés, con excelente fiabilidad y a concentración muy por debajo de la exigida por la legislación vigente. Del mismo modo, y tras las consiguientes modificaciones en el diseño, la utilización del microchip electrocinético puede plantearse como una atractiva alternativa para la monitorización electroquímica de numerosas muestras en paralelo, habitualmente realizado placas de microtitulación y con detección óptica.

Integración total de un método ELISA electroquímico en una plataforma microfluídica para la detección y control de niveles de ZEA en alimentos infantiles. *("Integrated electrokinetic magnetic bead-based electrochemical immunoassay on microfluidic chips for reliable control of permitted levels of zearalenone in infant foods")*

La posibilidad de miniaturización de los sistemas inmunoanalíticos en dispositivos de microfluídica, combina la alta sensibilidad y especificidad característica de la interacción antígeno-anticuerpo con las ventajas anteriormente mencionadas que proporcionan los sistemas miniaturizados (reducción en el consumo de reactivos, disminución del tiempo de análisis o la integración de múltiples procesos en un único dispositivo). Adicionalmente, mediante el empleo del fenómeno electrocinético se pueden manipular los fluidos de manera creativa y sofisticada a través de microcircuitos internos con el fin de llevar a cabo diferentes aspectos del análisis. Por todo ello, el objetivo de esta última etapa metodológica y como no podía ser de otra manera, fue el estudio de las posibilidades de integración de las etapas inmunoanalíticas en un sistema microfluídico de diseño sencillo, con el fin de utilizar el microchip como una plataforma sensora de nueva generación. La propuesta tuvo como finalidad desarrollar un sistema "lab-on-a-chip" que integrara todas las etapas del inmunoensayo en un único dispositivo y que permitiera llevar a cabo el control rápido, sencillo y reproducible de niveles permitidos de ZEA en alimentos infantiles.

La estrategia implica el uso creativo de un microchip sencillo de doble T, para llevar a cabo de forma secuencial e integrada las etapas inmunoanalíticas (inmunoreacción y reacción enzimática), y evitar así, los largos y laboriosos procedimientos asociados a los inmunoensayos convencionales. La reducción inherente de las distancias de difusión para los inmunoreactivos y la gran relación superficie/volumen incrementa las posibilidades de unión del antígeno y el anticuerpo dentro de los microcanales y reduce los tiempos de reacción.

La **Figura 21** ilustra la estrategia de integración basada en la separación física de la etapa de formación del inmunocomplejo y de la reacción enzimática en diferentes canales del chip,

mediante la adecuada manipulación de las partículas magnéticas (que actúan como soporte móvil de inmovilización). En este sentido se evita, de forma sencilla y elegante uno de los grandes problemas asociados a los inmunoensayos microfluídicos: la señal obtenida por la adsorción inespecífica debido al hecho de que la reacción enzimática se produce en una zona diferente a aquella donde tiene lugar la formación del inmunocomplejo.

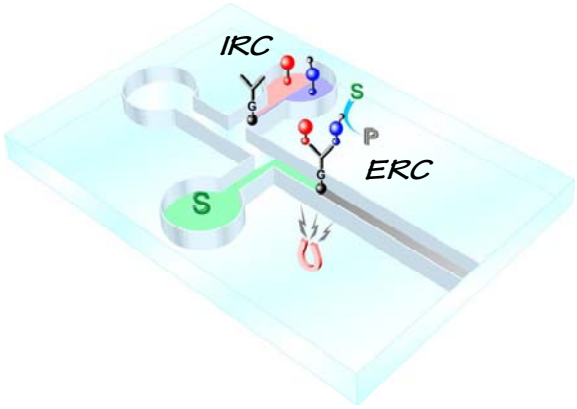


Figura 21. Representación de la estrategia de separación de las etapas inmunoanalíticas, (IRC: canal de inmunoreacción; ERC: canal de la reacción enzimática)

En primer lugar, y teniendo en cuenta las nuevas condiciones en las que se desarrolla la etapa de bioreconocimiento y reacción enzimática, se ha llevado a cabo la optimización de las variables que configuran el inmunoensayo microfluídico propuesto y en segundo lugar, la evaluación del método bajo el marco de la legislación vigente tomando como referencia las dos propiedades analíticas estudiadas en las aproximaciones anteriores: el límite de detección y la exactitud.

Del mismo modo que en las estrategias anteriores, el procedimiento analítico se basa en un ELISA competitivo en el cual las partículas magnéticas se utilizan como soporte de inmovilización y la enzima peroxidasa (HRP) se utiliza como marcador enzimático. La diferencia fundamental estriba en que ahora las etapas se realizan *"on-chip"*, por lo que se hace necesaria la re-optimización de todas las etapas inmunológicas. Únicamente la inmovilización del Ab sobre las partículas magnéticas se realiza *"off-chip"* (siguiendo el protocolo desarrollado en la estrategia 1). En efecto, las partículas magnéticas conteniendo a

los anticuerpos inmovilizados son introducidas en uno de los canales del microchip (canal de inmunoreacción, IRC) y retenidas mediante un imán. De esta forma, y una vez llenos los distintos reservorios y microcanales con las disoluciones adecuadas, mediante la aplicación de una combinación de campos eléctricos en los reservorios, se produce la inyección electrocinética de la disolución que contiene la muestra (ZEA) y una concentración conocida de ZEA-HRP, al canal de inmunoreacción. En el IRC, tiene lugar la competición entre ZEA y su conjugado enzimático por los sitios de unión con el anticuerpo. Posteriormente, las partículas magnéticas (con el inmunocomplejo formado) son manipuladas mediante un campo magnético externo, y llevadas a otro canal del microchip (ERC canal de la reacción enzimática), donde tras la inyección adecuada del substrato y el mediador desde el otro reservorio, tendrá lugar la reacción enzimática y la posterior detección electroquímica al final del canal, éstas últimas en las condiciones previamente optimizadas en la estrategia microfluídica anterior (artículo 3). El protocolo electrocinético y las condiciones optimizadas se han recogido de forma detallada en la Figura 2 (página 146, artículo 4).

Con el fin de demostrar la capacidad del inmunoanálisis microfluídico propuesto para la determinación de muestras contaminadas con ZEA, se ha construido la correspondiente curva de calibrado. El límite de detección obtenido (0.4 μg L^{-1}), aunque ligeramente superior al obtenido en las anteriores metodologías no microfluídicas, demuestra su validez, ya que se encuentra muy por debajo de los niveles permitidos por la legislación vigente (20 μg Kg^{-1}).

En este trabajo, con fines simplificar el procedimiento de calibración en el sistema microfluídico, se estableció otra estrategia de calibración simplificada basada en este caso en la comparación entre las señales del patrón y de la muestra. Para ello, la precisión del inmunoensayo microfluídico electroquímico también fue evaluada mediante el análisis de disoluciones control de ZEA a diferentes concentraciones (0, 1, 20 μg L^{-1}). La precisión *intra-ensayo* fue evaluada mediante cinco mediciones en el mismo nivel de concentración en cinco medidas consecutivas realizadas durante el mismo día. Esta serie de análisis se

repitieron durante cinco días distintos para estimar la precisión *inter-ensayo*. Mientras que los coeficientes de variación (CV) de los valores obtenidos para los niveles de concentración diferentes son aceptables para el mismo día, se puede observar un incremento de los coeficientes de variación obtenidos en días diferentes. Estas variaciones se pueden ver reducidas, si se utiliza un control (señal máxima, 0 μg L^{-1} (ZEA)) y los resultados se expresan como porcentaje en base al control. Este hecho es debido a que, aunque las señales absolutas varían para días diferentes, la relación entre las distintas señales y el control permanece constante, por ello se hace recomendable el uso de un control para mejorar los resultados analíticos.

El análisis del Material de Referencia Certificado, con objeto de determinar la exactitud, dio lugar a excelentes resultados, obteniéndose un intervalo de recuperaciones cercano al 100%, demostrándose así, la idoneidad del método propuesto.

Al igual que en los casos anteriores y tratando de demostrar la aplicabilidad de la metodología desarrollada, se realiza el análisis de ambas muestras representativas. Para ello, la papilla de cereales se fortifica con una concentración de ZEA igual a 20 μg kg^{-1}, la cual, después de las etapas de extracción y dilución correspondientes, alcanza una concentración final de 1 μg L^{-1}. Por esta razón, se utiliza una disolución estándar de ZEA de 1 μg L^{-1}, como referencia para estimar la presencia de ZEA en la muestra sólida. De esta forma, las muestras que produzcan corrientes menores o mayores que las producidas por la disolución de referencia (1 μg L^{-1}) serán consideradas como positivas o negativas respectivamente. El análisis de la papilla se realiza utilizando la calibración simplificada.

En el caso de muestras líquidas, directamente se toma como referencia la señal producida por una disolución de ZEA de 20 μg L^{-1} para considerar si las muestras analizadas son positivas o negativas (con respecto a la legislación vigente). De esta forma, el batido de cereales se fortificó con una concentración de ZEA de 20 μg L^{-1}, y se llevó a cabo su análisis directo.

Tanto en muestras sólidas, como en líquidas, los resultados obtenidos demuestran la aplicabilidad del inmunoensayo microfluídico para la determinación de la micotoxina al nivel máximo permitido por la legislación para alimentos infantiles.

Este trabajo demuestra que utilizando un microchip sencillo de doble T, y explotando la estrategia creativa de separación de las diferentes etapas del inmunoensayo en distintos canales del chip, se puede llevar a cabo el control de los niveles de ZEA en alimentos infantiles de forma segura mediante inmunoensayo electroquímico en menos de 15 minutos. Mediante esta estrategia, se evita el problema de la adsorción inespecífica de proteínas en el canal, a la vez que se reduce el tiempo total del análisis y se simplifica el procedimiento de calibración.

IV CONCLUSIONES

En esta sección se exponen las conclusiones derivadas de las distintas estrategias analíticas desarrolladas en esta Tesis.

Conclusiones

A partir de los resultados obtenidos en cada una de las estrategias propuestas y que se engloban dentro el inmunoanálisis electroquímico, se puede llegar a las siguientes conclusiones:

(i) El inmunoensayo desarrollado en placas de microtitulación, soportado en partículas magnéticas y con detección electroquímica, se configura como una herramienta válida y competitiva para la detección selectiva y sensible de ZEA en alimentos infantiles. Todo ello fundamentado en el LOD obtenido (0.011 μg L^{-1}), muy por debajo de los niveles permitidos por la legislación vigente (20 μg Kg^{-1}) y en la excelente fiabilidad del método en términos de precisión y exactitud. Sin embargo, el tiempo de análisis (90 minutos) y la laboriosidad del proceso de calibración podrían considerarse aspectos mejorables.

(ii) El inmunosensor electroquímico soportado en partículas magnéticas y empleando electrodos serigrafiados de carbono como superficie transductora, ha mostrado ser una herramienta altamente competitiva y en formato desechable, que permite simplificar el procedimiento de análisis de muestras mediante la calibración simplificada. Estas ventajas se alcanzaron sin detrimento de las excelentes propiedades analíticas obtenidas con un LOD del mismo orden que en la anterior estrategia (0.007 μgL^{-1}) y con excelente precisión y exactitud confiriéndole un alto valor añadido.

(iii) La combinación de plataformas microfluídicas con el inmunoanálisis electroquímico ha mostrado ser una novedosa y atractiva alternativa analítica para la determinación de ZEA en matrices alimentarias. En primer lugar, el sistema microfluídico ha evidenciado ser una excelente herramienta para llevar a cabo la monitorización electroquímica del inmunoanálisis desarrollado en placa de microtitulación. Asimismo, la integración de la calibración metodológica en el propio dispositivo evita los problemas asociados a la calibración convencional y simplifica el análisis de muestras reales. Hay que resaltar además, que los resultados

obtenidos aseguran la fiabilidad de la metodología desarrollada, tanto en términos de precisión como exactitud frente a un Material de Referencia Certificado de ZEA en maíz.

(iv) Dando respuesta al objetivo final de este trabajo de Tesis, la integración total del método ELISA electroquímico en un sistema microfluídico, ha mostrado ser una alternativa muy valiosa para el control de niveles permitidos de ZEA en alimentos infantiles. Los resultados obtenidos muestran igualmente su fiabilidad en términos de exactitud, precisión y límite de detección (0.4 μg L^{-1}). Del mismo modo, y a través de una sencilla y creativa estrategia en la que se usan microchips de doble T comercialmente disponibles, se ha reducido el tiempo total del análisis (menos de 15 minutos) y se ha simplificado el procedimiento de calibración.

Conclusions

This Thesis covers the development of different electrochemical immunoassay strategies to control permitted levels of zearalenone in food samples. After a detailed review of the results, some concluding remarks should be addressed:

(i) Conventional microtiter plate immunoassay, based on magnetic beads and electrochemically detected, has shown to be a very suitable and competitive tool for the sensitive detection of ZEA in baby food. The obtained limit of detection (0.011 μg L^{-1}), well below the legislative requirements (20 μg Kg^{-1}), together with a good reliability of the method in terms of precision and accuracy, allows a proper determination of ZEA in real samples. However, a long analysis time (90 minutes) and the laborious calibration process should be improved.

(ii) The electrochemical immunosensor based on magnetic beads and carbon screen printed electrodes (CSPEs), associated to a novel simplified calibration and analysis strategy has demonstrated to be an important, cost-effective and disposable alternative for high sensitivity detection and accurate determination of ZEA in baby food. These benefits were achieved without detriment to the excellent analytical properties obtained, where a good LOD (0.007 μg L-1) similar to the obtained in the first strategy, as well as an excellent precision and accuracy have shown the suitability of the proposed strategy.

(iii) The electrochemical immunoassays coupled to microfluidic platforms have also proven to be a novel and attractive alternative for ZEA determination in food samples. Firstly, the microfluidic system has shown its advantageous capabilities as analytical tool for electrochemical monitoring of the microtiter plate immunoassay developed in the first strategy. The integration of sequential and fast methodological calibration simplifies the conventional calibration procedure for performing the analysis of real samples. Moreover the results have shown its reliability in terms of precision and accuracy against a CRM in maize.

(iv) Secondly, a lab-on-a-chip strategy integrating all different analytical steps related to the biological recognition and electrochemical detection has been performed. The results have also shown its reliability in terms of accuracy, precision and detection limit (0.4 mg L-1). Likewise, through a simple and creative strategy, and just using a commercially available double T-microchip, the total assay time is reduced (less than 15 minutes) and the calibration procedure simplified.

V REFLEXIÓN FINAL

En esta sección se realiza una reflexión final sobre los resultados obtenidos en esta Tesis doctoral.

Reflexión final

Las conclusiones principales de este trabajo de investigación, se han ido detallando de forma específica en cada uno de los artículos publicados y de forma resumida se han incluido en el capítulo anterior. No se trata, en consecuencia, de rescatar el detalle relacionado con las optimizaciones y evaluaciones analíticas particulares, sino como continuación natural de la discusión conjunta, de establecer unas conclusiones generales. Sería, ciertamente interesante que, el establecimiento de tales conclusiones, produjera una reflexión conceptual y final que dé alcance y visión de los objetivos alcanzados y, por ello, permita contextualizar y evaluar en su conjunto, el trabajo desarrollado en esta Tesis Doctoral.

En una aproximación holística, se puede decir que las metodologías analíticas propuestas y desarrolladas en esta Tesis, todas ellas basadas en inmunoanálisis electroquímico soportado sobre partículas magnéticas para la detección y el control de la micotoxina zearalenona, (ELISA en placa, ELISA sobre electrodos serigrafiados de carbono y ELISA integrado en sistema microfluídico) constituyen en sí mismas una importante alternativa a las metodologías analíticas existentes. En efecto, la detección electroquímica no sólo supone una alternativa a la detección óptica en el marco del inmunoanálisis para la detección de esta micotoxina, sino que todas ellas, suponen una alternativa conceptual al empleo de técnicas analíticas de separación bien establecidas acopladas a espectrometría de masas en tanto que dan una respuesta químico-analítica plena acorde a los requerimientos más exigentes de la sociedad. Todas las metodologías inmunoanalíticas propuestas en esta Tesis, permiten la detección fiable de esta micotoxina en límites muy inferiores a los exigidos por la legislación más restrictiva (20 μg Kg^{-1}) demostrándose asimismo, la exactitud mediante el análisis de un material de referencia certificado. Adicionalmente, se ha demostrado que la detección y el control de zearalenona en muestras de alimentos infantiles elegidas como representativas de la problemática, se puede llevar a cabo de manera fiable debido a la excelencia de los resultados analíticos de recuperación obtenidos, todos ellos próximos al 100% con independencia de la matriz ensayada (sólida o líquida) y del procedimiento establecido de preparación de muestra para su análisis (extracción y/o dilución). Estos resultados nos indican a su vez, la extraordinaria calidad tecnológica tanto del anticuerpo

anti-ZEA como del conjugado enzimático empleados, aspecto que resulta crítico para obtener éxito cuando se emplean este tipo de metodologías.

Por otra parte, se han desarrollado con éxito estrategias miniaturizadas sin detrimento alguno de las prestaciones analíticas inherentes del inmunoanálisis electroquímico para la detección y el control de zearalenona. Estas estrategias miniaturizadas se han diseñado con la base común de desarrollar plataformas inmunosensoras de diferente abordaje: inmunodetección sobre electrodos de carbono serigrafiados e inmunodetección integrada en un sistema microfluídico electrocinético, empleando tecnologías serigrafiada y "lab-on-a-chip", respectivamente.

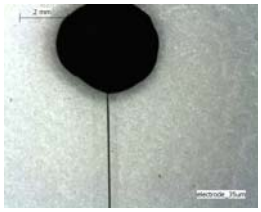
En este sentido, los electrodos serigrafiados de carbono se han configurado como una valiosa herramienta para la construcción de inmunosensores electroquímicos para la detección de zearalenona empleando volúmenes de muestra de tan solo 40µL y cuya principal ventaja ha sido que pueden resultar desechables.

Por su parte, la tecnología "lab-on-a-chip" ha permitido obtener dos hallazgos importantes. Por una parte, ha demostrado su capacidad analítica para monitorizar el ELISA en placa y por otra, ha permitido la integración de todas las etapas requeridas para llevar a cabo el inmunoanálisis (bio-reconocimiento e incubación enzimática) en una plataforma microfluídica electrocinética de diseño sencillo. El éxito de esta integración surge del empleo creativo de los diferentes microcanales de las plataformas, así como de las ventajas inherentes que nos proporciona la manipulación de fluidos para llevar a cabo operaciones analíticas en la micro escala. Esta integración identifica diferentes hallazgos importantes tales como evitar la indeseable adsorción inespecífica y presentar inherentemente una elevada versatilidad en lo que a la manipulación electrocinética de fluidos se refiere; en nuestro caso, a través de la aplicación de campos eléctricos. Asimismo, dado que el diseño está disponible comercialmente evita la necesidad de sofisticadas instalaciones tales como una habitación blanca, haciéndola accesible a cualquier laboratorio, lo que la configura en la escena analítica contemporánea como una plataforma inmunosensora de nueva generación para la detección y control de esta micotoxina.

Por otra parte, en todas las estrategias miniaturizadas y como alternativa a los tediosos procedimientos de calibración característicos de los inmunoensayos, se han propuesto y desarrollado con éxito diferentes protocolos de calibración metodológica simplificada y/o integrada. Todos ellos, después de estudiar en profundidad el procedimiento clásico de calibración basado en el establecimiento de la función log-log*it* de cuatro parámetros, se basan en definir adecuadamente los factores de calibración que permiten, en todos los casos, la simplificación del proceso, el empleo de menor número de patrones y de tiempo. Es preciso resaltar que la evaluación analítica de las metodologías que involucraron estos protocolos de calibración simplificada fue re-ensayada, arrojándose de nuevo excelentes resultados de exactitud y límites de detección; siendo en consecuencia, de fiable aplicación para el análisis de las matrices alimentarias infantiles.

Por todo ello, sin pretender ser ambiciosos y desde un punto de vista crítico pero realista, se puede concluir que estas estrategias miniaturizadas suponen un valor añadido a las prestaciones analíticas inherentes y demostradas del inmunoanálisis electroquímico, debido a que permiten alcanzar las bondades analíticas mencionadas de fiabilidad y LODs adecuados en términos mejorados de simplicidad, disminución de reactivos y muestras, rapidez y disminución de coste. Sin embargo, quizá sea necesario resaltar que uno de los aspectos más sobresalientes de las mismas sea que suponen un avance importante hacia la descentralización y simplificación de los análisis; configurándose como nuevas herramientas analíticas en el ámbito de la seguridad alimentaria.

VI ANEXO/ANEXX



Microfabrication and testing of carbon microelectrodes for use in lab-on-a-chip applications

MICROFABRICATION AND TESTING OF CARBON MICROELECTRODES FOR USE IN LAB-ON-A-CHIP APPLICATIONS

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Period of stay: from 1st June to 15th September 2009

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1. INTRODUCTION

Electrochemical detection offers a great promise for many lab-on-a-chip applications, with features that include remarkable sensitivity, inherent miniaturization, independence of sample turbidity and optical path length, low cost and high compatibility with microfabrication technologies. These characteristics make electrochemistry suitable for integration in miniaturized devices. Furthermore, miniaturization of electrodes does not diminish the analytical performance and most of the methods used for fabrication of microfluidic network can also be employed to construct integrated electrodes[1,2].

The microfabrication process is defined as the fabrication of devices in which at least one dimension is in the micrometer scale. This process is usually based on the deposition of different layers on a substrate, called wafer. The wafers can be made from different materials, being silicon, glass and ceramics the most used. The electrical and mechanical properties of silicon make it an indispensable material for thin film technology.

The application of this technology in the fabrication of electrochemical microtransductors is relatively recent[3], being a consequence of the introduction of micro-TAS or "lab-on-a-chip" concepts by A. Manz et al., in the early nineties[4]. From this starting point, production has experienced significant growth.

These devices have some advantages over conventional electrodes[5], such as low production cost, its reduced size without losing of performance, high compatibility with micro fabrication techniques used for "lab-on-a-chip" devices, mass production and, the ability to work online, *in vivo* or *in vitro*. However, its production needs from special laboratories called clean rooms, where strict control of the temperature, humidity, pressure and particles in the air is required. The contamination risk through airborne particles is prevented by the use of continuous air filter.

Regarding to **microfabrication techniques**, broadly two approaches termed thin and thick film technologies are found. By one hand, in thin film technologies, typically, during the fabrication process of the microfluidic device, a thin metal film is deposited in vacuum onto the substrate using a shadow mask, which projects a geometrical pattern onto the substrate.

Lithography and lift off process[6,7], which involves photoresist and further development steps, sputtering[8] and evaporation[9] are among these type of technologies. Gold and platinum are often used for thin film metallization. On the other hand, the fabrication methods for thick film microelectrodes obviate the need of clean room facilities and costly apparatus for vacuum deposition. Common thick film microelectrodes employing carbon and metal materials can be found in the form of screen printed electrodes (SPEs)[10], electrodes created by airbrushing and micromolding.

When the microfabrication is involving a polymer, the main machining approaches are replication from a master (moulding methods) and direct machining. Replication methods often produce a microestructure by allowing a polymer workpiece to form an inverse copy of a mould. The formation of microchannels using moulding methods generally involves two steps: (i) master fabrication, and (ii) channel transfer moulders to polymer substrates. On the other hand, direct machining methods remove small amounts of polymers in places where the microstructures should be located (laser ablation). A particular example of polymer machining of great significance is the poly(dimethylsiloxane) (PDMS). This polymer is a widely used polymer substrate for soft lithography-based microfabrication and its major advantage is the ability of rapid prototyping of complex structures. Channels in PDMS are easily formed by replica moulding, which is simply the generation of a negative replica from the master in PDMS.

A deep description of these microfabrication techniques would be obviously outside of this chapter, although the main outlines will be briefly described.

Thin film technology: photolitography

Photolitography is the most used microfabrication technique. This process let transfer different designs present in a mask to a wafer. Briefly, the first step is the deposition of a material called photoresist, onto the wafer, which changes its solubility when is irradiated with ultraviolet light. Once it has been irradiated following the design of the mask, it is immersed in a solution suitable to remove non-polymerized photoresist, thereby transferring the design from the mask to the wafer surface[11]. There are two types of

photoresist, depend on its reaction with UV light[12] as it is depicted in **Figure 1.** In *positive photoresist*, the incident-UV light areas become soluble and are eliminated in the development process, transferring the geometric design on the wafer. In the *negative* one, the incident- UV light areas polymerize and become more resistant in the development process, being not incident-UV light areas removed.

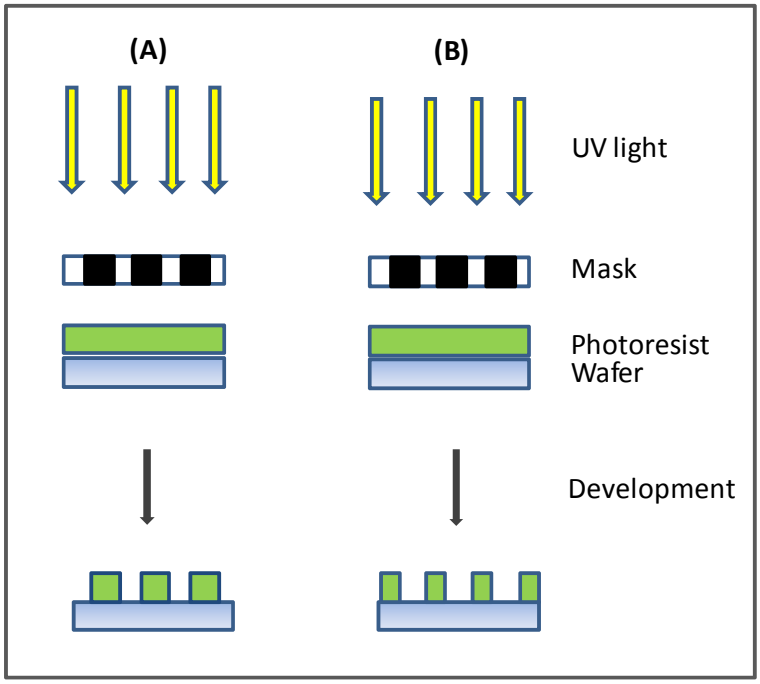

Figure 1. Photolitography process involving positive (A) and negative (B) photoresists.

Thin film technology: Lift off

Lift off or indirect etching technique is used for some metals that are difficult to make the etching by other techniques. This process involves three basic steps as it is shown in **Figure 2**. In the first step, the photoresist is deposited using photolithographic techniques, defining the needed design to manufacture the device. Then, material (metal or dielectric) is deposited onto the wafer that will form the thin layer. Finally, the wafer is immersed in a bath of a solvent, usually acetone, able to dissolve the photoresist and leave only the material deposited on it.

In consequence, it could be said that thin film microfabrication technology allows the incorporation of multiple electrodes on-chip by the deposition of different metals (gold, carbon...) on the channel. It requires the use of expensive metallization and clean room

facilities, besides integration of more than one electrode material is challenging. Nevertheless, thick film technology becomes easier to integrate due to its non-dependence of clean room facilities.

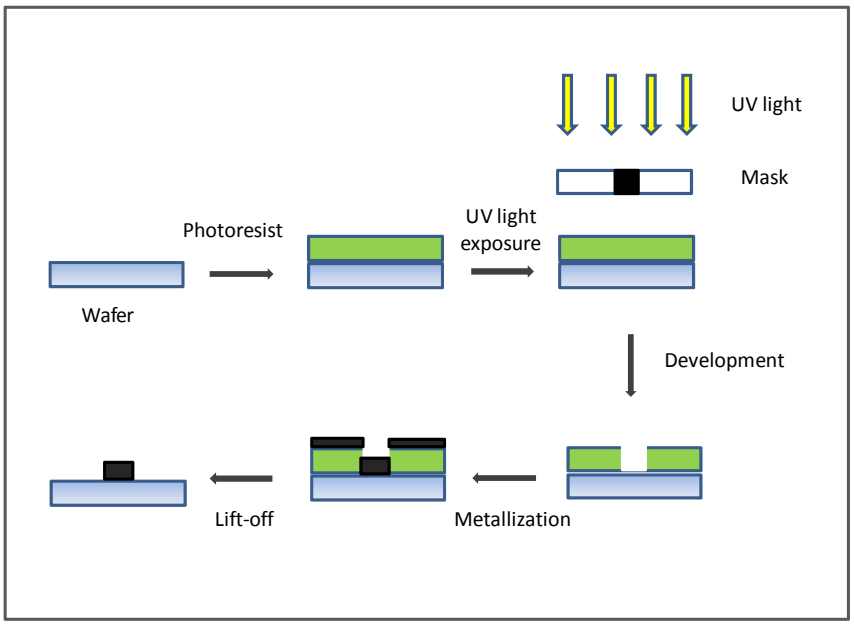

Figure 2. Scheme of the lift-off process.

Carbon microelectrodes fabrication: background

In electrode microfabrication, the most common materials used are carbon, platinum, and gold. While platinum and gold offer narrow potential window employing amperometric detection, carbon-based materials are used due to its features such as the wide potential window, low background currents, resistance to fouling by organic redox couples, low cost, solvent compatibility, high corrosion resistance, and good electric conductivity.

With respect to carbon microelectrodes, patterned carbon electrodes have been created using pyrolyzed photoresist, carbon ink, and screen-printing. Many researchers utilize commercially available carbon inks (CIs) for sensing applications. The main attracting features of CI are the reasonable cost and the wide potential window for sensing. Also, most of the reported carbon electrodes are fabricated by depositing carbon ink on a solid substrate (*screen printing technique*)[13].

One of the most important contributions have been proposed and developed by Martin´s group. Indeed, this group has successfully incorporated thick film carbon ink microelectrodes on microchips through a micromolding technique for various biological investigations[14-20]. In general sense, this method uses PDMS microchannels that can be reversibly sealed to a glass substrate to define the size of the microelectrode. The fabricated microelectrodes usually were electrochemically characterised and then, used for amperometric detection on microfluidic devices for different biological applications. The introduction of the use of these new electrodes by this group was proposed in 2004[15]. In this work, (PDMS)-based microchannels to define the dimensions of the microelectrodes was proposed where microchannels are filled with the appropriated solution of carbon ink. The resulting microelectrodes fabricated by micromolding technique were properly characterized by microchip-based flow injection analysis exhibiting a good analytical performance (good linearity, LODs of 2mM). Also, carbon microelectrodes fabricated by this PDMS micromolding using an *off-channel* electrochemical detection were also evaluated by the same group[17]. In this work, in conjunction with micromolded carbon ink electrodes for the detection of catecholamines, a Pd decoupler was used. The hybrid PDMS/glass device was characterized with fluorescence microscopy and by monitoring the CE-based separation of dopamine as model analyte. Other novel application of Martin´s group was the fabrication method for 3-dimensional fluidic device containing PDMS-embedded microelectrodes that individually address each layer and enable simultaneous detection in the fluidic channels[18]. The PDMS-embedded carbon microelectrodes were fully characterized with catechol and the ability to increase the selectivity of the electrode was demonstrated by detection of nitric oxide, a key small molecule messenger that plays an important role in a variety of biological process.

The characterization of these electrochemical transducers was usually carried out by cyclic voltammetry using model analytes with a reversible redox behavior. This technique allows knowing quickly the redox behaviour of chemical species in a wide range of potentials. Cyclic voltammetry provides qualitative and quantitative information, parameters such as, oxidation and reduction potentials, kinetic aspects of electron transfer and mass on the surface of the transducer. The most used model is constituted by the redox system

$Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$. This is a well known reversible redox system, for which the difference between E_a (anodic peak potential) and E_C (cathodic peak potential) is $\Delta Ep = 59 / n$ mV for a good electron transfer of the electrode (this value is independent of scan rate)[21,22].

2. MOTIVATION

The aim of this work has been the microfabrication and testing of carbon microelectrodes for using in lab-on-a-chip applications. Electrode microfabrication was performed using two approaches; micromolding and lift-off techniques.

(i) The first approach was based in a micromolding technique using poly-(dimethylsiloxane) (PDMS)-based micro channels as a mould to define the pattern and dimensions of the fabricated microelectrodes. Afterwards, the formed microchannels were filled with the appropriated solution of carbon ink.

(ii) In a second approach, photolithography was used to construct the master with the required electrode designs, followed by spin-coating of the appropriated carbon ink solution, and a final lift-off step.

3. RESULTS AND DISCUSSION

Micromolding technique for fabrication of carbon microelectrodes

The desired layout was initially drawn using the software "CleWin" and printed with high resolution on a transparency sheet, resulting in the corresponding photomask. In **Figure 3** are depicted the prepared micromolding channels, where it can be observed variations in width (35, 50, 100 and 150 µm), thickness (7-40 µm), and keeping 2 cm long for all of them.

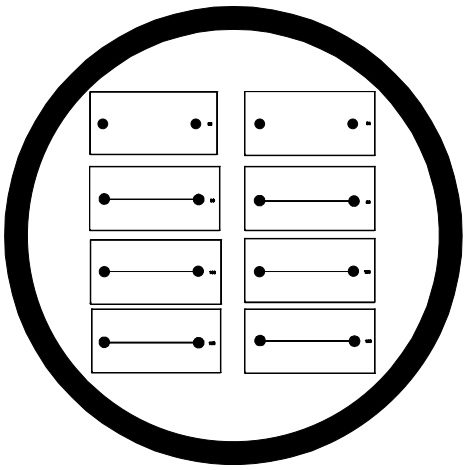

Figure 3. Photomask with different micromolding channel width (2x35µm, 2x50 µm, 2x100 µm and 2x150 µm) (black circle is indicating the mask shape).

The corresponding masters were prepared following the procedures described in the section **Clean-room protocols,** using the positive or negative photoresist, as a function of the desired thickness.

The hybrid microedevices were fabricated using PDMS and microscope glass slides. The glass layer will contain the carbon microelectrodes patterned according to the microchannels-mould produced in the PDMS layer. An inlet and outlet were also produced in the PDMS microchannel. To prepare the PDMS layer, a mixing elastomer and curing agent in a 10:1 ratio with a total weight of 44 g was made. Then the mixture was poured in a Petri-dish containing the master wafer and it was cured on a hot plate at 75 ºC for approximately 2 hours. Next, the PDMS microchannels were removed from the master, cut

and placed on the microscope glass slide with channel facing down as presented in **Figure 4**.

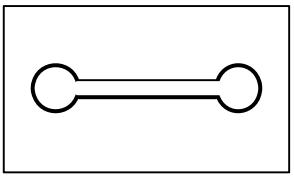

Figure 4. PDMS micromolding channel for carbon microelectrodes fabrication.

In the preparation of carbon ink microelectrodes, the PDMS microchannels were sealed with a microscope glass slide (that had been cleaned with Piranha solution (H_2SO_4/H_2O_2 70:30% (v/v)), and primed with solvent thinner (N-160) for approximately 1 min. Next, the thinner was removed by applying a vacuum to one of the reservoirs. A mixture of commercially available carbon ink and solvent thinner was added to the channels and pulled through the channel by applying vacuum to the opposite end. The ink/thinner mixture was made so that the volume of added thinner was 0.2% (v/w) of the initial carbon ink weighted. After filling microchannels with carbon ink solution, the entire chip was placed in a hot plate at 75ºC for 1 h. After this time, the PDMS molds could be removed from the glass, leaving the carbon microelectrode attached to the glass surface. A final curing step was achieved by placing the microscope glass slide containing the carbon microelectrode in a hot plate at 120ºC for 1 h. Connections to the carbon microelectrode were made by using copper wire and silver glue. Schematic of the method used to fabricate the carbon microelectrodes is shown in **Figure 5**.

Several studies were done to improve the fabrication of the carbon microelectrodes prepared with this methodology. As we have already mentioned before, first, the PDMS micromolding channels with different electrode widths (35, 50, 100 and 150 µm) and different thickness (7 and 40 µm) were reversibly sealed to a glass substrate. It was found that the wider and deeper microchannels were the easier to fill it with the carbon ink solution. It was also found, than the resulting carbon microelectrodes adhered better to the glass if it was previously cleaned with piranha solution.

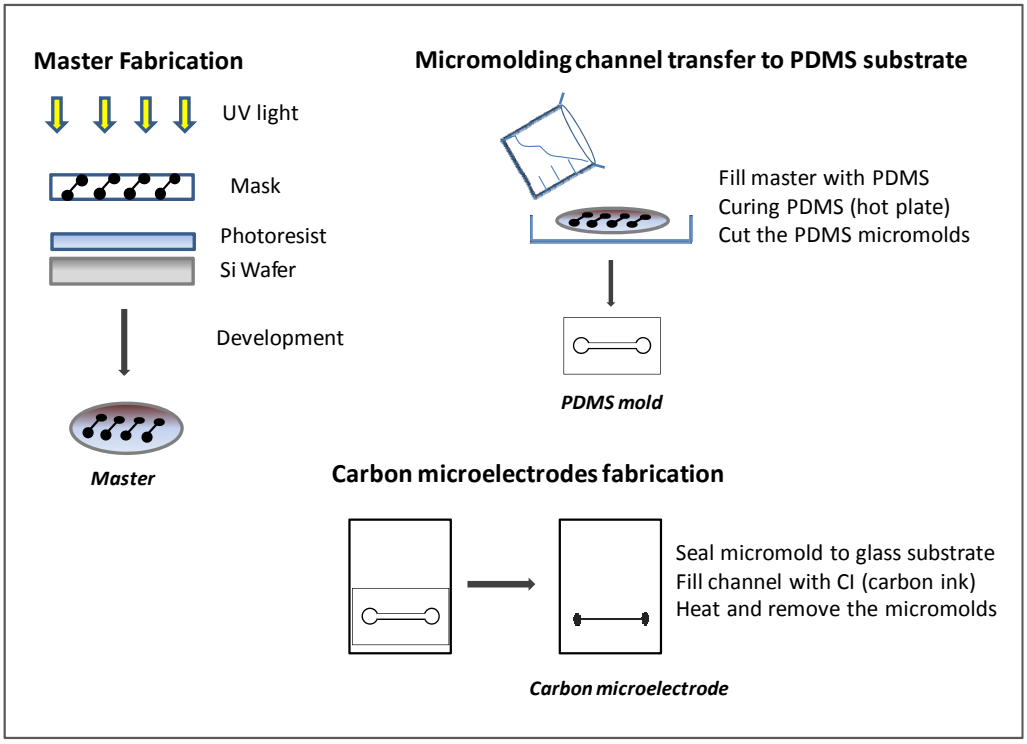

Figure 5. Steps for the fabrication of carbon microelectrodes by micromolding technique.

Afterwards, the micromolding channels were primed with a thinner solution, left in the channel for 1 minute, and subsequently removed by vacuum. If this step was omitted, filling the channel with the ink was not successful. The commercially ink employed in this work was very viscous and could not be handled unless it was diluted with the thinner solution. A variety of mixtures were tested, where 0.2 % (v/w) was found to be optimum for filling the PDMS microchannels.

After heating at 75ºC for 1 h, the PDMS molds could be peeled up from the glass, leaving behind the carbon microelectrodes. A final heating step at 120ºC was used to pretreat the electrode (removing solvents from the electrode and promote cracking to increase the effective surface area). **Figure 6** shows a picture of the final microelectrode obtained.

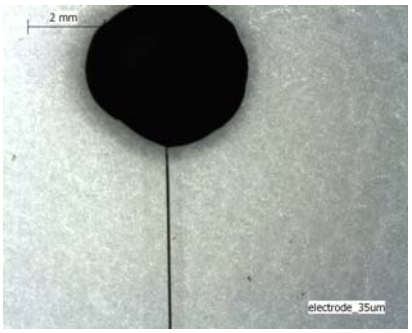

Figure 6. Picture of a carbon microelectrode (35 µm wide, 7 µm deep and 2 cm long) prepared by micromolding technique.

The carbon microelectrodes were characterized by cyclic voltammetry of the reversible redox system ferri/ferrocyanide. The results showed a slow electron transfer with a ΔEp value considerably higher than the theorist value of 59 mV for this redox system (for 35 µm, ΔEp= 630 mV and for 150 µm, ΔE= 780 mV).

Photolitography and lift-off technique for fabrication of carbon microelectrodes

As in the previous procedure, the desired lay out was initially drawn using the same software "CleWin" and printed with high resolution on a transparency sheet, resulting in the corresponding photomask similar to shown in **Figure 3,** but now with different widths of the carbon microelectrodes (2x35µm, 2x50 µm, 2x75 µm, 2x100 µm, 2x150 µm, 2x200 µm, 2x300 µm, 2x500 µm, 2x1000 µm and 2x1500 µm) and 7 µm thickness.

The masters were prepared following the procedure described in the section **Clean-room protocols** for the positive photorestist AZ 2070, where different substrates as silicon and glass wafers together with different cleaning methods were tested. Once the master was prepared, the carbon ink solution was added on the middle of the wafer, which was put into a spin-coater. The appropriate program was applied to the wafer (see section Clean-room protocols for the positive photorestist AZ 2070) and after the spinning, lift-off with 1-methyl-2-pyrrolinone (NMP) in the fume hood was carried out. Connections to the carbon microelectrode were made by means of a copper wire and silver glue.

Schematic of the accomplished procedure is described in **Figure 9**. The mask was designed with different electrode widths (100, 150, 200, 300, 500, 1000 and 1500 µm), while the master was prepared using different wafers: silicon and glass. Then, the master was spin-coated with the appropriated solution of carbon ink, and the lift-off was made with NMP.

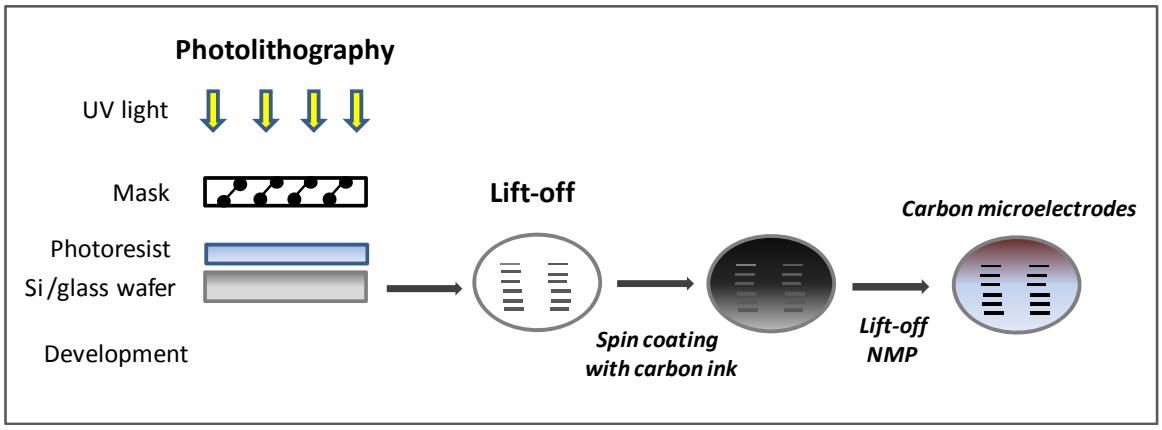

Figure 9. Fabrication process for carbon microelectrodes by photolitography and lift-off technology.

To characterize the electrochemical properties of the prepared carbon microelectrodes, cyclic voltammetry analysis using the reversible redox system ferri/ferrocyanide was also conducted. **Table 1** lists the results obtained with the lit-off approach. Although, for the prepared electrodes, the ΔEp values remained higher than the predicted value of 59 mV, a better behavior was observed when silicon wafer was used because of their inherent properties.

Finally, we can conclude that carbon microelectrodes with different dimensions using micromolding and photolithography lift-off techniques were successfully fabricated. The results of the carbon microelectrodes prepared by micromolding technique showed a slower electron transfer that those obtained by photolithography and lift-off technique. Also, when photolithography and lift-off technique was used, silicon wafers showed better electron transfer than glass ones. To the best of our knowledge, this is the first time that a photolithography technique, that uses spin-coating of the carbon ink for the metal deposition and lift-off, is used to fabricate carbon microelectrodes. This route is very promising to be integrated in novel "lab-on-a-chip" devices.

Table 1. Electrochemical characterization of carbon microelectrodes fabricated by lift-off technique.

Width (μm)	**ΔEp[1] (mV)**	**ΔEp[2] (mV)**
1500	263	257
1000	380	208
500	362	167
300	365	170
200	329	191
150	427	189
100	449*	-

[1] ΔEp was calculated using ferri/ferrocyanide (1 mM in KCl 0.1 M) redox system measured with the carbon microelectrodes prepared in **glass wafers**. Glass wafers cleaning with UV-ozone (15 min) and hard bake at 150ºC (30 min); (*) Glass wafer cleaning with piranha and hard bake at 150ºC (30 min)

[2] ΔEp was calculated using ferri/ferrocyanide (1 mM in KCl 0.1 M) redox system measured with the carbon microelectrodes prepared in **silicon wafers**. Silicon wafers cleaning with HF etching.

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5. MATERIALS, EQUIPMENTS AND CLEAN-ROOM PROTOCOLS

Materials and Reagents

- Silicon and glass wafers (4 inch).
- SU-8 50, AZ 2070 photoresists.
- Developer SU-8 and Developer AZ 826MIF (tetramethyl- ammonium hydroxide solution).
- N-methyl-2-pyrrolidone (NMP).
- PDMS (SYLGARD 184) Silicone elastomer and curing agent Dow Corning.
- Ercon E-978 (I) carbon ink and N-160 solvent thinner (Ercon, Wareham, MA).
- Potassium Ferrocyanide $K_3Fe(CN)_6 \cdot 3H_2O$.

All reagents used were of analytical grade.

Equipment

- A Spin coater CEE model 100 made the deposition of the photoresist.
- An UV radiator OAI (Optical Associates Inc.) model 30/5; time exposure OAI 150; illumination controller 2105C2, was used to make the UV-light exposure through the corresponding masks.
- An Ultraviolet ozone cleaning system: UVOCS, model T10X16/OES/MOD (series 270) was used for the cleaning process of the glass wafers.
- The bake steps were carried out by a Hot-plate.
- A Microscope Leica S6D, was used to check the microchannels.
- The cyclic voltammograms were performed by a Potentiostat HCH Instruments, Electrochemical Analyzer, CHI832B. A homemade Reference electrode Ag/AgCl (tip pipette with silver wire filled KCl 3M).

Clean-room protocols

Lithography wafer with negative photorestist SU-8-50 (40 µm thickness)

In order to fabricate the master for the PDMS micromolding channels, the following steps were carried out.

- Silicon Wafer cleaning

  1. Rinse the wafer once with acetone and dry it with N_2 gun.
  2. Rinse the wafer once with isopropanol and dry it with N_2 gun.
  3. Rinse the wafer once with milliQ Water and centrifuge it dry.
  4. Heat wafer for 5 minutes at 150ºC on the hot plate, and then cool down till room temperature.

- Spin-coat step with CEE™ –100 spincoater

  1. Centre the wafer on the chuck of the spincoater by using the spincoat program and then start it.
  2. Add slowly 4.5 ml SU-8 on the middle of the wafer, avoiding air-bubbles.
  3. Use the spin coat program (500 rpm, 13 s; 875 rpm, 44 s).

- Pre-exposure bake step

  1. Put spin-coated wafer on the hot-plate.
  2. Heat the wafer (from room temperature to 65º C in 25 min; 10 min at 65ºC; from 65ºC to 95ºC in 15 min; incubate for 30 min at 95º C and cool down to room temperature).

- Exposure step; OAI UV exposure system (365 nm)

  1. Switch on the UV-lamp and heat the lamp for 20 min.
  2. Measure output of the lamp with borofloat wafer.
  3. Put the wafer under the lamp and cover it with the mask.

4. Avoid movement of the mask during exposure, putting a borofloat wafer on the mask.

5. Expose the wafer with doses of 250 mJ/cm: once 20 s and once 5.6 s.

- Post exposure bake

1. Put the wafer on the hot-plate.

2. Heat the wafer:

   a) From room temperature to 70º C in 75 min; incubate for 60 min at 70º C.

   b) From 70º C to 90º C in 15 min; incubate 5 min at 90ºC.

   c) Cool down to room temperature.

- Development step

1. Put the wafer in a glass petri-dish with SU-8 developer.

2. Move the wafer from time to time to remove non-exposed SU-8 (around 5 minutes).

3. Put the wafer in fresh developer and incubate it for 5 min more.

4. Check if rinsing with iso-propanol completes the development. (If there is non-exposed SU-8 on the wafer, the wafer or parts of the wafer will be white, then incubate it in SU-8 developer for 5 min).

5. If all non-exposed SU-8 is removed then dry it and put it in a box.

Lithography wafer with positive photorestist AZ 2070 (7 µm thickness)

In order to fabricate the master for carbon microelectrodes by photolithography and lift-off technique, where the master defines the size of the carbon microelectrodes, the following steps were carried out.

- Silicon wafer cleaning: Etching with HF.

1. Immerse the wafer in a HF solution.

2. Immerse the wafer in water (for eliminating HF residues).
3. Rinse the wafer once with milliQ water and centrifuge until it dry.
4. Heat the wafer for 5 minutes at 102ºC on the hot plate and then cool down until room temperature.

- Glass Wafer cleaning: UV-Ozone cleaning:

1. Cleaning the wafer with UV-Ozone for 15 minutes.
2. Heat wafer for 30 minutes at 150 ºC on the hot plate; clean with N_2 (g) gun and cool down until room temperature.

- Spincoat step with CEETM –100 spincoater

1. Centrate the wafer (silicon or glass) on the chuck of the spincoater.
2. Add slowly 4.5 ml AZ 2070 on the middle of the wafer; avoid air-bubbles.
3. Use the spincoat program:
    a) Program 0: Velocity: 500 rpm; Ramp: 100 rpm/s; Time: 13 s
    b) Program 1: Velocity: 875 rpm; Ramp: 300 rpm/s; Time: 44 s (1 min per micrometer of photoresist).

- Pre-exposure bake step

1. Leave the spincoated wafer 15 minutes at room temperature in horizontal way.
2. Put spincoated wafer on the hot-plate.
3. Heat the wafer:
    a) From room temperature to 102ºC and incubate it for 7 min at 102ºC (softbake).
    b) Cool down to room temperature.
    c) Clean the wafer with N_2 gun.

- Exposure step: OAI UV exposure system (365 nm)

1. Switch on the UV-lamp and heat the lamp for 20 min.

2. Measure output of the lamp with borofloat wafer.
3. Put the wafer under the lamp and cover it with the mask.
4. Avoid movement of the mask during exposure, putting a borofloat wafer on the mask.
5. Expose the wafer with doses of 250 mJ/cm: 20 s.

- Post exposure bake

1. Put the wafer on the hot-plate.
2. Heat the wafer:
    a) At 112º C for 1 minute (postbake).
    b) Cool down to room temperature.
3. Hardbake: heat the wafer at 150 º C for 1 hour.

- Development step

1. Put the wafer in a glass petri-dish with AZ developer.
2. Move the wafer from time to time till all non-exposed AZ is solved (around 1 minute).
3. Put the wafer in fresh developer; incubate for 1 min.
4. Check if rinsing with water completes the development.
5. If all exposed AZ is removed then dry it and put it in a box.
6. Check the channels through the microscope.

Procedure preparation ink solution and spin coating over the wafer

1. Weigh about 6 gram of carbon ink in a flask and add 0.738 ml solvent/gram ink.
2. Close the flask and shake very well.
3. Take the flask to the cleanroom.
4. Spin-coat the ink over the wafer at 3000 rpm for 30 seconds (same program as photoresist).

5. Put the wafer on a hot-plate and increase the temperature from room temperature to 102º C in about 15 min (on ceramic plate).

6. Incubate the wafer at 102º C for 1 hour.

7. Lift off with NMP in the fume hood.